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# **Identification and Characterisation of Novel Shellfish Allergens for Improved Diagnosis**

Thesis submitted by

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M.Sc. (Biotechnology)

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**James Cook University**

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### Chapter 2:

**Kamath SD**, Abdel Rahman AM, Komoda T, Lopata AL. Impact of heat processing on the detection of the major shellfish allergen tropomyosin in crustaceans and molluscs using specific monoclonal antibodies. *Food Chemistry* 2013; 141:4031-9.

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### Chapter 3:

Abdel Rahman AM, **Kamath S**, Lopata AL, Helleur RJ. Analysis of the allergenic proteins in black tiger prawn (*Penaeus monodon*) and characterisation of the major allergen tropomyosin using mass spectrometry. *Rapid Communications in Mass Spectrometry* 2010; 24:2462-70.

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of Newfoundland

**Chapter 4:**

**Kamath SD**, Abdel Rahman AM, Voskamp A, Komoda T, Rolland JM, O'Hehir RE, Lopata AL. Effect of heat processing on antibody reactivity to allergen variants and fragments of black tiger prawn: A comprehensive allergenomic approach. *Molecular Nutrition and Food Research* 2014; 58:1144-55.

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## Chapter 6:

Abramovitch JB, **Kamath S**, Varese N, Zubrinich C, Lopata AL, O'Hehir RE, Rolland JR. IgE Reactivity of Blue Swimmer Crab *Portunus pelagicus* Tropomyosin, *Por p 1*, and Other Allergens; Cross-Reactivity with Black Tiger Prawn and Effects of Heating. *PLoS ONE* 2013; 8:e67487.

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## Chapter 7:

**Kamath SD**, Thomassen MR, Saptarshi SR, Nguyen HM, Aasmoe L, Bang BE, Lopata AL. Molecular and immunological approaches in quantifying the air-borne food allergen tropomyosin in crab processing facilities. *International Journal of Hygiene and Environmental Health* 2014; 217:740-50.

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**Sandip D. Kamath**

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This PhD Thesis is dedicated to,

My parents, *Maya D. Kamath* and *Dayanand B. Kamath*,  
and to my wife, *Shruti S. Kamath*

॥ॐ श्री गणेशाय नमः॥

**Abstract:**

Food allergy is in general defined as a type I, IgE antibody mediated hypersensitivity reaction, which is triggered on exposure to innocuous food sources. Increased consumption of seafood has not only resulted in higher incidences of allergic reactions but also a rise in occupational allergic sensitisation and asthma among workers due to inhalation of air-borne food allergens. The problem of accidental allergen exposure is compounded by the highly cross-reactive nature of major shellfish allergens with dust mite and insect allergens.

A detailed review on the current status of food allergy and diagnostic approaches is provided in **Chapter 1**. In the case of crustacean and mollusc allergy, allergy testing is mostly performed using whole crude shellfish preparations. The crude protein extracts currently used for such tests are prepared using only a few selected European species which are not commonly consumed in the Asia-Pacific region. Such diagnostic tests may not be accurate enough to detect specific IgE against allergens present in shellfish species specific to Australia. Moreover, since most of the crustaceans are processed by means of heat-treatment, the effects of heat on crustacean allergens and subsequent IgE binding properties are not fully understood.

The work presented in this PhD thesis provides a comprehensive study on the identification and characterisation of major and novel allergens found in shellfish species, specific for the Asia-Pacific region and their relevance among the Australian population. Furthermore, the effects of heat processing on the stability of shellfish allergens and the molecular basis of immunological cross-reactivity were investigated.

In **Chapter 2**, an extensive panel of raw and heat-treated shellfish allergen extracts were produced which included eleven crustacean and seven mollusc species. Tropomyosin was identified as the major heat-stable allergenic protein in the tested shellfish extracts using specific monoclonal antibodies. Enhanced antibody binding was demonstrated to tropomyosin in all heat-treated crustacean species but only in few selected mollusc species. Based on the antibody binding data and worldwide importance, Black tiger prawn was

investigated in further detail for characterisation of the prawn allergen repertoire and the effects of heat-processing on their IgE binding properties.

In **Chapter 3**, novel mass spectrometric methods were developed and validated for the identification and sequencing of tropomyosin and other shellfish allergens. Subsequently in **Chapter 4**, IgE binding proteins in the Black tiger prawn raw and heat-treated extracts were detected using sera from a patient cohort. Several IgE binding allergens were identified in addition to tropomyosin such as arginine kinase, myosin light chain, sarcoplasmic calcium binding protein, triose-phosphate isomerase and two putative novel allergens fructose bis-phosphate aldolase and titin. Enhanced IgE binding was observed to prawn allergens in the heat-treated protein extracts. For the first time, IgE binding was demonstrated to a heat-generated tropomyosin fragment which highlighted the stability of the allergenic molecule post-degradation and which may have implications in exposure to heat-processed prawns. Three-dimensional homology modelling of the allergens highlighted the existence of dimeric or oligomeric protein structures; shown to be a strong characteristic of allergenic proteins.

In **Chapter 5** and **Chapter 6**, the immunological cross-reactivity of tropomyosin was analysed in detail between three commonly consumed Australian crustacean species; Black tiger prawn, King prawn and Blue swimmer crab. Differential IgE antibody binding was observed to King prawn tropomyosin as compared to Black tiger prawn tropomyosin thus indicating structural variation of the IgE binding epitopes. Moreover, higher IgE reactivity was observed to the heated prawn or crab extracts in patients sensitised to tropomyosin, thus demonstrating enhanced IgE reactivity to tropomyosin post heat treatment. Basophil activation assays revealed stronger IgE reactivity to raw prawn and crab extracts among patients who lacked IgE binding to tropomyosin, thus indicating reactivity to crustacean allergens other than tropomyosin. To further investigate the differential IgE binding observed for crustacean tropomyosin, a multiple sequence alignment of 60 invertebrate tropomyosins was performed specifically for the previously identified eight IgE binding epitopes. A detailed comparison revealed epitope-specific conservation or variation among the various allergenic tropomyosins. IgE binding epitope regions 2 (amino acid

residue 91-101), 4 (187-197) and 5a (251-259) were found to be highly conserved among crustacean and mollusc species and may be responsible for the immunological and clinical cross-reactivity observed.

Finally, in **Chapter 7** a sensitive immunoassay was developed and validated for the quantification of aerosolised crab tropomyosin in crab-processing factories. Using this sensitive assay, tropomyosin was quantified in the personal breathing zones of 80 crab processing workers. High exposure levels to airborne crab allergens were observed among workers involved in crab meat-boiling or heating activities, thus indicating higher generation of bio-aerosols in these areas.

In summary, tropomyosin seems to be a relevant cross-reactive major allergen among the affected Australian population. Heat-processing of shellfish has an enhancing effect on the IgE antibody binding and reactivity of tropomyosin and other heat-stable allergens. *Ex vivo* IgE reactivity was demonstrated to the identified prawn allergens, highlighting the importance of characterising the whole allergen repertoire in other shellfish species. The work presented in this thesis provides an important contribution towards the development of improved and sensitive allergy diagnostic platforms. Future work involving IgE epitope mapping of the identified shellfish allergens is central to the development of component resolved diagnostics and immunotherapeutic strategies for shellfish allergy.

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## LIST OF ABBREVIATIONS

°C – Degree Celsius

µL – Microlitre

AK – Arginine kinase

ANOVA – Analysis of variance

BLAST – Basic Local Alignment Search Tool

BTP – Black tiger prawn

cDNA – Complementary deoxynucleic acid

DBPCFC – Double blinded placebo controlled food challenges

ELISA – Enzyme-linked immunosorbent assay

FBA – Fructose bis-phosphate aldolase

g – gravity

HRP – Horseradish peroxidase

IgE – Immunoglobulin Isotype E

IgG – Immunoglobulin Isotype G

kDa – Kilodaltons

M – Molar

mg – Milligram

mL – Millilitre

MLC – Myosin light chain

MWCO – Molecular Weight Cut Off

ng – Nanogram

ORF – Open reading frame

PBS – Phosphate buffered saline

PBZ – Personal breathing zone

PCR – Polymerase chain reaction

PFF – Peptide fragment fingerprinting

pg – picogram

PMF – Peptide mass fingerprinting

PTM – Post translation modification

PVDF – Polyvinylidene difluoride

RNA – Ribonucleic acid

RPM – Revolutions per minute

RT-PCR – Reverse transcriptase – polymerase chain reaction

SCBP – Sarcoplasmic calcium binding protein

SDS – Sodium dodecyl sulphate

SPT – Skin prick test

TBS – Tris buffered saline

TIM – Triose phosphate isomerase

TM – Tropomyosin

# CHAPTER 1

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## INTRODUCTION TO SHELLFISH ALLERGY AND DIAGNOSTICS

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## 1.1 Introduction to shellfish allergy

Shellfish is an important source of food and plays a significant role in human nutrition and health. The last decade has witnessed an increase in the worldwide consumption of various seafood products mainly due to changed perceptions of dietary requirements as a result of an increase in awareness of the health benefits of seafood. This in turn has resulted in a growing international trade in shellfish species and products, adding to the popularity and frequency of consumption in many countries.<sup>1</sup> In Australia alone, it is estimated that seafood consumption will increase from 12 kg per year per person to nearly 23 kg per year till 2050.<sup>2</sup> More importantly, seafood and in particular shellfish are considered as a significant source of food required to feed an ever increasing human population in the future. Black tiger prawns are one of the most widely cultured and consumed prawns in the world with an annual production of 900,000 tonnes.<sup>3</sup> However, this increase in the consumption and production has been accompanied by a rise in the incidences of adverse reactions to shellfish in both consumers and seafood processors, respectively.

Shellfish allergy is a long lasting disorder which mostly persists throughout life and is often associated with severe reactions.<sup>4</sup> In the USA and Canada, shellfish allergy is one of the most commonly reported cause of food induced anaphylaxis and visits to the emergency departments.<sup>5, 6</sup> The allergenic group of shellfish can be broadly classified into crustaceans and molluscs. Of all the various consumed shellfish, prawns are one of the most widely consumed crustacean group and causes the most severe reactions.<sup>7</sup>

Recent population-based studies conducted on food allergy have demonstrated a rise in the prevalence of shellfish allergy. A telephone-based survey conducted in the USA showed 2 % of the general population are affected by shellfish allergy.<sup>8</sup> This prevalence figures, however, seem to vary according to different geographical regions as shown by a Singapore-based study where 5.3% of children were shown to be affected by this disease.<sup>9</sup> In Australia, a study conducted by Turner *et al* demonstrated that 25% of children with definite clinical reaction to seafood were allergic to shrimps.<sup>10</sup>

A systematic approach to diagnosis requires a careful examination of history linked to an understanding of the clinical manifestations, understanding the epidemiology and immune cause, and incorporation of various test results.<sup>11</sup> Depending on the medical history, minimally invasive tests such as skin prick test (SPT) or oral food challenges (OFC) are used to determine a high probability of allergic sensitisation.<sup>12</sup> However, allergists appear to avoid performing OFCs, because of the health risks to the patients, cost, time and other factors.<sup>13</sup>

*In vitro* diagnosis, in contrast, is performed by quantification of allergen-specific IgE antibodies. However, crude preparations of shellfish extracts are mostly used in these tests, which do not represent the exposure to various shellfish species consumed in that region, and may lead to errors in specific diagnosis and identification of the offending species. Various allergenic proteins can trigger reactions to shellfish, and it is important to identify and characterise these macromolecules. Understanding the structure and function of these allergens is important not only to the understanding of IgE reactivity in patients and the underlying mechanism but also to the development of sensitive and improved diagnostic platforms. Recent advances in the field of molecular cloning and recombinant technology have enabled the production of purified recombinant allergens that have myriad potential in the field of component resolved diagnostics and allergen quantification.

Exposure to shellfish allergens can also occur through inhalation leading to occupational allergy and asthma. This is an important aspect of shellfish allergy in terms of occupational health and safety where seafood processing workers are constantly exposed to air-borne allergens in bioaerosols generated during the processing activities. It is therefore essential to develop sensitive techniques for the detection and monitoring of these air-borne allergens for better management of occupational allergy.

In this thesis, the major allergens found in shellfish species, which are most commonly consumed in Australia have been identified and characterised. In depth analysis and allergen characterisation was conducted for the Black tiger prawn; one of the most consumed and allergy provoking shellfish species in the

Asia-Pacific region. Furthermore, the effects of heat-processing on IgE antibody binding and cross-reactivity of shellfish allergens were analysed in detail. Novel analytical techniques and sensitive immunoassays were developed and validated for the identification and quantification of the major shellfish allergens.

## 1.2 Classification of seafood

Several seafood species consumed in different regions of the world are often identified using different common names. Patients may fail to identify the offending source, due to the confusion regarding the use of different common names for different species of seafood. The most important commonly consumed seafood can be generally classified into chordates (fish), arthropods and molluscs. The phylum chordata includes bony fish (Osteichthyes) such as cod, tuna and barramundi. However, the allergens identified in fish are very different from invertebrate allergens and shall not be further discussed. The two invertebrate phyla of arthropods and molluscs are generally termed as “shellfish” which are further categorised (Figure 1.1).

Edible arthropods belong to the class crustacea and more specifically to the order decapoda. This group includes prawns, shrimps, crabs and lobsters. Most crustaceans have 5 pairs of legs on the main thoracic body and 5 pairs of swimmerets on the abdomen or tails.<sup>14</sup> Crustaceans are closely related to arachnids (dust mites, spiders etc.) and insects such as cockroaches. Among the edible crustaceans, prawns constitute a major part of the consumed and farmed species. In Australia, the production of prawns, wild-catch and aquaculture, were nearly 23,000 tonnes in 2011-12 with a value of \$172 million. Prawn production is mainly carried out in Queensland, Western Australia and South Australia.<sup>3</sup> Prawn species such as Black tiger prawn, King prawn and Banana prawn are commercially important in Australia. On an international scale, the top prawn producers are China, India, Indonesia, Thailand, Taiwan and the Philippines, which account for 75% of the world production. Seafood imports to Australia are predominantly from China, New Zealand, Vietnam and Thailand.<sup>2</sup>

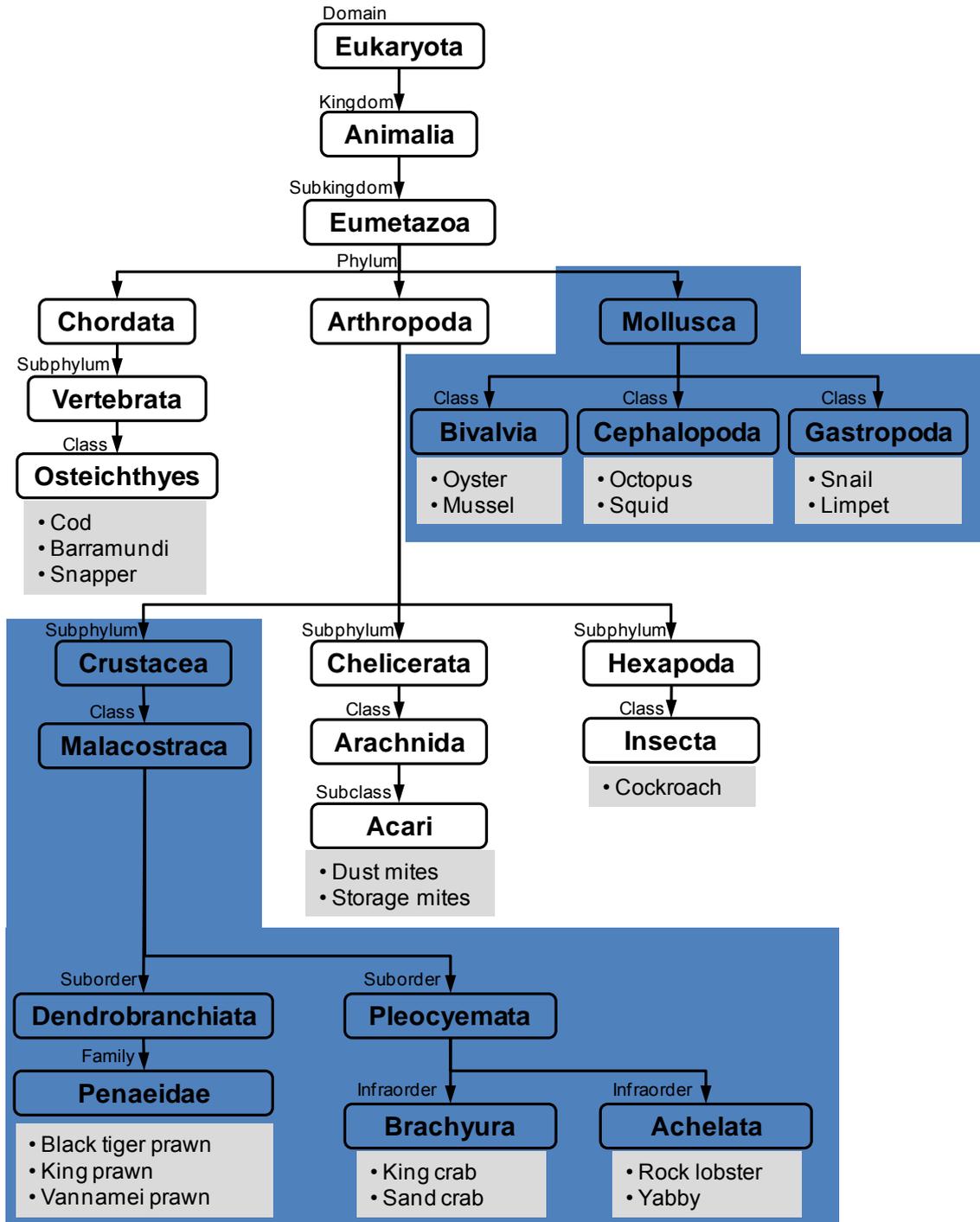
Decapods from the Penaeidae family are termed as prawns and those from the Caridae family are called as shrimps. Prawns and shrimps thus belong to two different taxonomical classifications with the main anatomical differences being the different overlapping pattern of the segments in the carapace and their brooding methods.<sup>15</sup> Penaeids are larger in size compared to shrimps and commercially important. Most of the species tested and characterised for their

allergens and prevalence belong to this family. Shrimps are smaller in size and used mainly as additives or flavouring agents. Common examples of the caridean shrimps are the freshwater shrimp, *Macrobrachium rosenbergii*, and northern shrimp, *Crangon crangon*.

However, the term “prawns” and “shrimps” are often used interchangeably in the commercial industry as well as in various research studies. The term prawns are used in Australia and other commonwealth countries whereas the term shrimp is most commonly used in the USA and Europe. The general use of prawns and shrimps is often confusing in the scientific literature, since it tends to address the same crustacean species. For example, *Penaeus monodon* has been mentioned as Black tiger prawns<sup>16</sup> or Black tiger shrimps.<sup>17</sup> Similarly, *Litopenaeus vannamei* is referred to as vannamei prawns or white pacific shrimps.<sup>18</sup> Nevertheless, these studies are specific to prawn allergies.

Mollusca are also a large and diverse group with over 100,000 different species currently identified. Commercially important mollusc species are broadly classified into three categories. Bivalve includes mussel, oyster, clam and scallop; gastropod includes snail, abalone and limpet and cephalopod includes octopus and squid (Figure 1.1).

Although crustaceans and molluscs, which constitute the majority of the consumed shellfish, are taxonomically different and diverse in nature, clinicians often advise for complete avoidance of both the groups to allergic patients. This is partly due to the cross-reactive nature of some allergenic proteins found in shellfish, although this is not a common occurrence.<sup>14</sup>



**Figure 1.1:** Classification of major seafood groups and related invertebrate species. The blue shaded regions indicate the different consumed species commonly termed as “shellfish”. The texts shaded in gray provide examples for each seafood or invertebrate group.

### 1.3 Adverse reactions to food

Adverse reactions to food are an important problem faced in today's fast changing world due to increased food consumption and changes in dietary habits. Different food processing methods have led to the inclusion of various food allergens in unexpected forms, and may cause accidental adverse effects in affected people.

Adverse reactions to food can be divided into toxic reactions, non-immune mediated food intolerance and immune-mediated food allergy such as IgE-mediated hypersensitivity and non-IgE mediated reactions. IgE mediated immune reactions are the major contributor of adverse reactions to shellfish.

#### 1.3.1 Food allergy

Allergy can be defined as an adverse immune response to innocuous antigens which are not associated with invading pathogens.<sup>19</sup> Allergic sensitisation and hypersensitivity can occur on exposure to various sources such as food, inhalant or aeroallergens, venom and drugs. The onset of allergy has been shown to depend on several factors such as genetic susceptibility, route of exposure, allergen load and allergenic protein structural characteristics. In the 1920's, it was shown that allergen sensitivity could be transferred from an allergic to a non-allergic individual by injection of the serum.<sup>20</sup> Later in 1967, the serum factor responsible for the sensitivity was identified as IgE antibodies.<sup>21, 22</sup> IgE antibodies are normally produced in response to parasitic infections, but in the case of allergic reactions, they are produced against non-pathogenic macromolecules.<sup>23</sup> IgE antibody mediated hypersensitivity is the most common form of allergy. This form of allergy affects more than 25% of the general population in industrialised nations.<sup>24</sup>

Food allergy is an adverse immune response to food proteins and is responsible for a variety of symptoms involving the gastrointestinal tract, respiratory tract and skin.<sup>19</sup> Recent studies have indicated a rise in the incidences of food induced allergies, with as many as 6% of children and 3% of adults being currently affected by it.<sup>25</sup>

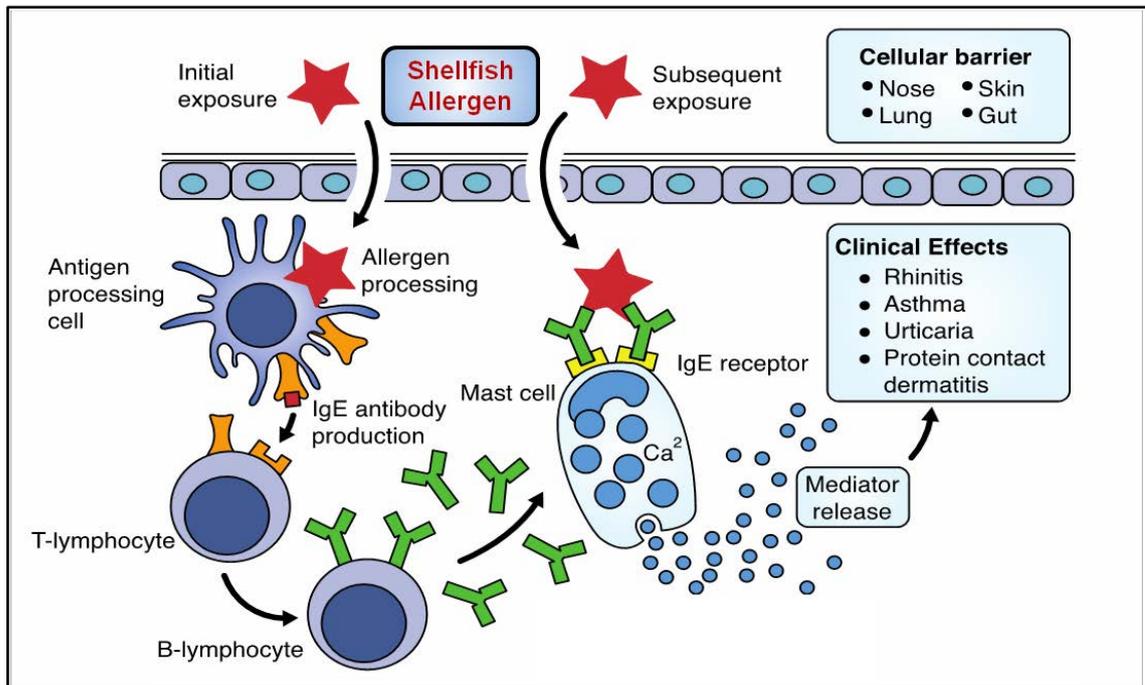
### 1.3.2 Mechanism of IgE-mediated Type I hypersensitivity

During sensitisation to an allergen, the specific CD4<sup>+</sup> T helper 2 cells (T<sub>H</sub>2) produce cytokines such as interleukin 4 (IL4) and interleukin 13 (IL13). These events are responsible for the class switching to  $\epsilon$  immunoglobulin heavy chain, thus leading to the production of immunoglobulin E (IgE) antibodies by the B cells.<sup>26</sup> These allergen-specific IgE antibodies sensitise mast cells and basophils by binding to the high affinity receptor (Fc $\epsilon$ RI), which are expressed on the cell surface. On exposure to the allergen source, a type I hypersensitive reaction is triggered when the allergens are able to bind and cross-link the cell-surface bound IgE. (Figure 1.2) On cross-linking of the IgE-Fc $\epsilon$ RI complexes, the mast cells and basophils degranulate, releasing vasoactive amines, lipid mediators, chemokines and other cytokines, all of which characterise the immediate phase of an allergic reaction.<sup>26</sup> IgE can also bind to the low affinity receptor, Fc $\epsilon$ RII, which is also known as CD23 on the B cell surface. This increases the uptake of the allergenic proteins by antigen presenting cells for presentation of the allergen-peptides to CD4<sup>+</sup> T cells, which leads to the late phase of an allergic reaction.<sup>27</sup>

IgE antibodies were first declared as the fifth isotype of immunoglobulins in 1968.<sup>28</sup> IgE shares the same structure as the other isotypes with two identical light chains and two identical heavy chains. However, the heavy  $\epsilon$  chain consists of one more domain as compared to the  $\gamma$  chain in IgG.<sup>26</sup> In a healthy individual, IgE antibodies are normally produced in response to parasitic infections. However, in atopic individuals, IgE is produced in response to normally innocuous antigens.<sup>29</sup> IgE antibodies are present in the serum in relatively lower concentrations as compared to the other isotypes, and have a short serum half-life of 2 days.

The cross-linking of cell surface bound IgE on basophils and mast cells trigger the degranulation and release of several pre-formed mediators. Among these is histamine, a vasoactive amine which causes an increase in the local blood flow and vessel permeability.<sup>27</sup> Enzymes are also released such as chymase, tryptase and serine esterases, which cause a breakdown of tissue matrix proteins and tissue damage. High amounts of tumour necrosis factor (TNF)- $\alpha$

are also released which promotes the influx of leukocytes and lymphocytes into the tissues.<sup>27</sup> Apart from the release of pre-formed mediators, mast cells also synthesize and release chemokines, lipid mediators such as leukotrienes and additional cytokines such as IL-4 and IL-13, which perpetuates the  $T_H2$  response. The lipid mediators cause an immediate smooth muscle relaxation, increased vascular permeability and mucus secretion.



**Figure 1.2:** Schematic representation of a Type I, IgE-mediated hypersensitivity reaction<sup>30</sup>

### 1.3.3 Routes of exposure and clinical manifestations of allergic reaction

Food allergens can elicit an IgE mediated reaction by first penetrating through the skin, gut or respiratory lining by exposure through skin contact, ingestion or inhalation, respectively. The stereotypic symptoms of IgE-mediated reactions are rapid in onset, which can result in multi-system or systemic manifestations. In general, IgE-mediated reactions are considered to be acute reactions; however they are also associated with chronic symptoms through late-phase reactions.<sup>31</sup>

Allergic symptoms can be categorised into five groups; Generalised reactions (anaphylaxis), Respiratory reactions (Asthma and rhinitis), Cutaneous reactions (urticaria, angioedema or atopic dermatitis), Gastrointestinal reactions (Abdominal pain, nausea, vomiting and diarrhoea) and other reactions such as conjunctivitis and oral allergy syndrome. A summary of the different implicated seafood species and clinical manifestations are summarised in Table 1.1.

#### 1.3.3.1 Anaphylaxis

Anaphylaxis is the most severe form of an IgE-mediated allergic reaction to food.<sup>32</sup> It is estimated that there are 30,000 cases of food-related anaphylactic reactions, treated in emergency departments every year in the United States.<sup>33</sup> Anaphylaxis is a rapid multisystem IgE mediated reactions that can be fatal. The onset is rapid, often in seconds to minutes of food ingestion. In some cases, hypotension has been reported as the primary symptom of anaphylaxis. Fluid extravasation and vasodilation can lead to a decrease in circulating blood volume of up to 35% within 10 minutes.<sup>34</sup> Currently, there is no reliable test for the diagnosis of anaphylaxis. Tryptase, released by mast cells during the reaction, is not consistently elevated in food anaphylaxis.<sup>35</sup>

#### 1.3.3.2 Respiratory symptoms

Upper and lower respiratory tract symptoms manifest as rhinoconjunctivitis, laryngeal oedema, and asthma. Inhalational exposure to food allergenic proteins in occupational settings such as shellfish processing factory activities, and non-occupational settings like restaurants, school, and airliners may affect

individuals with specific food allergy. Asthma is one of the most commonly reported reactions to food by inhalation.<sup>36</sup>

### **1.3.3.3 Cutaneous symptoms**

Cutaneous manifestations of allergic reactions are typically acute and consist of urticaria or angioedema. Urticaria upon contact of allergens with skin is a common symptom and must be differentiated from irritant and allergic contact dermatitis. Urticaria lesions are defined as chronic if manifestations recur over a period of 6 weeks.<sup>37</sup> Contact dermatitis occurs from handling foods and is typically observed as a manifestation of occupational allergy.<sup>38</sup> Raw seafood is one of the most implicated foods for contact dermatitis. The affected skin area is eczematous, with erythema and vesiculation.<sup>39</sup>

### **1.3.3.4 Oral allergy syndrome**

The oral allergy syndrome (OAS) is the most frequent clinical presentation of food allergy seen in adult patients.<sup>40</sup> OAS is characterised by the rapid onset of oral pruritus after the ingestion of shellfish. Other symptoms associated with OAS are burning and oedema of lips, tongue, palate, and throat. These symptoms start within minutes of eating the offending food.<sup>41, 42</sup> In most cases, the symptoms of OAS do not progress to anaphylaxis.<sup>32</sup>

**Table 1.1:** Classification of seafood groups causing allergies, representative species and common symptoms.

	Phylum	Class	Name	Symptoms	Allergens
<b>Shellfish</b>	<b>Arthropods</b>	Crustaceans	prawns, lobster, rock lobster, crab, barnacle	<ul style="list-style-type: none"> <li>• Urticaria</li> <li>• GI symptoms</li> <li>• Larygoedema</li> <li>• Urticaria</li> <li>• Oral allergy syndrome (OAS)</li> <li>• Rhinitis</li> <li>• Asthma</li> <li>• Anaphylaxis</li> </ul>	<ul style="list-style-type: none"> <li>• Tropomyosin</li> <li>• Arginine kinase</li> <li>• Myosin light chain</li> <li>• Sarcoplasmic calcium binding protein</li> </ul>
			Gastropods		abalone, snail, limpets
	<b>Molluscs</b>	Bivalves	Clam, oyster, mussel, cockles		<ul style="list-style-type: none"> <li>• Tropomyosin</li> <li>• ?</li> </ul>
			Cephalopods		Squid (cuttlefish), octopous
<b>Fish</b>	<b>Chordates</b>	Bony fish	Salmon, hake, tuna, herring, carp		<ul style="list-style-type: none"> <li>• Parvalbumin</li> </ul>

### 1.3.4 Toxic reactions and food intolerance

Non-immune reactions can be triggered by a range of substances, including marine toxins and biogenic amines. Additional ingredients such as preservatives, flavours and colourings added during processing of shellfish can cause adverse reactions. Such ingredients include chemical additives (e.g. sodium benzoate; metabisulphites), spices (e.g. mustard, flour additives and garlic) and some such as casein which are not always obvious (hidden ingredients).<sup>43, 44</sup>

#### 1.3.4.1 Toxins

##### Shellfish Poisoning by Marine Algae Toxins

The majority of toxin related adverse reactions to shellfish are however caused by toxin producing algae. These toxin producing algae form often large blooms that change the colour of the water and are called “Red Tide”. These algae are usually taken up by filter feeders such as mussels and oysters but also abalone and squid. These toxins accumulate in the tissue and in turn are consumed by humans, demonstrating symptoms similar to seafood allergy.<sup>45</sup> Importantly, the large variety of toxins cannot be destroyed by heating the shellfish before consumption.

##### PSP Toxins

Among the phycotoxins, PSP (Paralytic Shellfish Poisoning) has been recorded very frequently and accounts for the majority of human fatalities. Minute dinoflagellates (algae) produce over 20 different toxins including the well-characterised neurotoxin Saxitoxin, which blocks the neuronal and muscular sodium channels. Symptoms include gastrointestinal signs but may include prickly sensations in the fingertips and toes and even choking sensation and muscular paralysis.

##### DSP Toxins

Diarrheic Shellfish Poisoning encompasses several toxic components including Okadaic acid. Chronic exposure to DSP toxins may also promote tumour

formation in the digestive tract. Symptoms are mainly of a gastrointestinal nature such as diarrhoea, nausea, vomiting and abdominal pain.

#### **1.3.4.2 Bacterial and viral contamination**

Shellfish poisoning outbreaks have frequently been associated with bacterial contamination of shellfish harvested in waters containing inadequately treated sewage. Primarily different *Vibrio* strains such as *V. cholerae* or *V. vulnificus* have been implicated as well as *Listeria* and *Salmonella* species. In many cases of poisonings after consumption of raw shellfish, small round structured viruses (SRSVs) were associated, as well as hepatitis A.<sup>46, 47</sup>

#### **1.3.4.3 Seafood intolerance**

Food intolerance involves non-immune mechanisms and often implicates gastrointestinal problems as well as severe headaches or rash. Symptoms may be caused by pharmacological properties of the ingested seafood such as histamine and tyramine (Scombrototoxicity), which can be found in large amounts in canned and pickled fish but less frequently in shellfish. In addition food colourings such as tartrazine and other azo dyes and food preservatives such as sulphites and benzoates have been implicated as well as spices (e.g. mustard, paprika, flour additives, garlic). Food flavourings such as monosodium glutamate (MSG) can trigger the so-called 'Chinese restaurant syndrome', also found in shellfish products. MSG can provoke asthma and tingling sensation in the limbs and face. In shellfish intolerance reactions a dose responsive effect is usually demonstrated and confirmation is best performed by DBPCFC testing.

## 1.4 Prevalence, epidemiology and distribution of shellfish allergy

Shellfish is one of the “Big Eight” food groups (Egg, Milk, Wheat, Soy, Peanuts, Tree nuts, Fish and Shellfish), which accounts for 90% of all food allergy cases. It is generally considered that shellfish and fish are among the top four food groups responsible for provoking severe food anaphylaxis.<sup>8, 48, 49</sup>

The prevalence and distribution of shellfish allergy depends on the geographical regions due to changing dietary habits and consumption of seafood as the staple diet.<sup>50</sup> Several risk factors are proposed to influence food allergy or sensitisation, including sex, ethnicity, genetics, atopy, vitamin D insufficiency, increased hygiene and the timing and route of exposure to foods.<sup>19, 25</sup> Accurate determinations of food allergy prevalence are difficult because of factors such as allergy definitions, study populations, methodologies for allergy testing, geographic variations, ages and dietary factors.<sup>51</sup> Furthermore, accurate epidemiological data on the prevalence of shellfish allergy are limited by the lack of population-based data incorporating the double-blind placebo controlled oral food challenge (DBPCFC).<sup>1</sup> Although DBPCFC are considered to be the gold standard in diagnosing food allergies, this test is time-consuming and it is not frequently used in clinical care as many children are diagnosed on the basis of history and other test results.<sup>52</sup>

Several studies have been conducted to establish the prevalence of shellfish allergy based on confirmed clinical reaction, skin prick testing, food specific IgE quantification, random telephone surveys or self reported. A telephone survey conducted in the US including 14,648 participants demonstrated that adults seem to be more affected more than children by shellfish allergy with a prevalence of 2% and 0.3%, respectively. Of the identified participants with shellfish allergy, 38% and 49% have perceived allergies to crustaceans and molluscs, respectively with only 14% reacting to both shellfish groups.<sup>8</sup> In a randomised cross-sectional survey administered in US households, involving a total of 38,480 children, the prevalence of shellfish allergy was found to be 1.3%.<sup>53</sup> A study in Spain involving 355 children established that 6.8% of patients reacted to crustaceans by skin prick test.<sup>54</sup> A study from South Africa with perceived adverse reactions to seafood confirmed the sensitisation to

prawn and rock lobster. Of the 131 positive reactions by ImmunoCAP, 50% reacted to four crustacean species.<sup>1, 55</sup> A recent study from Australia involving 167 children with definite reaction to seafood established anaphylactic reaction in nearly one fifth of the patient cohort. Moreover, 25% of all seafood allergic children elicited reactivity to shrimps.<sup>10</sup> Another study conducted in Australia involving 2848 infants recruited during routine immunisation sessions, established that 0.9% of all tested children elicited reactivity to prawns.<sup>56</sup> In a recent meta-analysis conducted on the prevalence of common food allergies in Europe, the self-reported lifetime prevalence for shellfish allergy was 1.3% whereas the food challenge defined shellfish allergy was 0.1%. The heterogeneity between studies may be partly due to self-reporting of non-IgE-mediated adverse reactions. Hypersensitivity to shellfish was found to be more common in older children.<sup>57</sup>

Although shellfish allergy seems to be common in western countries such as the United States, Europe and Australia, the prevalence appears to be higher in Asian countries.<sup>58-61</sup> A study conducted in Singapore based on a structured written questionnaire which involved 25,692 school children demonstrated a prevalence of 1.2% in children aged 4-6 years and 5.2% in children aged 14-16 years.<sup>9</sup> A Hong Kong based study involving 3677 Chinese pre-school children aged 2-7 years incorporating parent reported and doctor diagnosed adverse food reactions, revealed shellfish to be the leading cause of allergy among 15.8% children.<sup>62</sup> In Asia, hypersensitivity to shellfish and fish was more common than to nuts, peanuts and wheat.<sup>63</sup> The likelihood of being sensitised to shellfish appears to correlate to the geographical eating habits, where seafood is part of the staple diet. Interestingly, the first intake of seafood appears to be very early in life, as early as 7 months, in the Asian community.<sup>58</sup>

In contrast to geographical and dietary factors influencing the prevalence of shellfish allergy, the racial and ethnic differences have not been explored widely. In a telephone survey, shellfish allergy was reported at a higher rate in the African American subjects as compared to the Caucasian subjects.<sup>8, 51</sup> In spite of the fact that several studies have been performed investigating the prevalence of shellfish allergy in different regions of the world, there is a paucity of data for the two most populous regions of the world; India and China.

Food induced anaphylactic reactions are an important health concern. It is estimated that 30,000 cases of food induced anaphylaxis are seen annually in the United States of which 200 are fatal.<sup>1</sup> Shellfish has been deemed the most common trigger in adults and regarded as persistent with a high risk of anaphylaxis.<sup>51, 64</sup> In a study conducted by Lau et al in the US, nearly half of the children with shellfish allergy had a history of severe reactions.<sup>53</sup> Similarly, shellfish is the leading cause of food induced anaphylaxis in South-East Asia, Hong Kong and Taiwan.<sup>9, 51, 65, 66</sup>

In general, childhood food allergies to milk, egg, wheat and soy usually resolve with age, where allergies to shellfish, nuts and fish persist into adulthood.<sup>25</sup>

## 1.5. Occupational allergy to seafood

Occupational allergy and asthma is a serious health concern also affecting seafood-processing workers. According to the Food and Agricultural Organisation (FAO), over 45 million people are involved in the fishery and aquaculture industry.<sup>67, 68</sup> The increase in consumption and subsequent increase in fishing and harvesting activities in the last three decades have been associated with exposure to allergenic seafood proteins, allergic disease and asthma. Workers in this industry are exposed to seafood, involved in manual or automated processing of crabs, prawns, mussels or fish.<sup>69</sup>

The aetiology and development of allergic diseases are due to the interactions between genetic, environmental and host factors which give rise to different allergic disease phenotypes.<sup>68-70</sup> Occupational allergy to seafood can manifest as both upper and lower respiratory symptoms, as well as urticaria and protein contact dermatitis. Rhinitis and conjunctivitis may also occur which may precede chest symptoms.<sup>68</sup> The prevalence of occupational asthma in seafood processing workers is between 2 and 36%.<sup>69-71</sup> About 7% of the workers with ingestion-related allergy develop asthma symptoms associated with inhalational allergen exposure.<sup>72</sup> Conversely, there are rare cases of workers with occupational asthma who subsequently developed ingestion-related allergic symptoms to the same seafood species.<sup>70</sup>

Exposure and inhalation of seafood allergens depends on the generation of bioaerosols during the manual or automated processing activities in the factories. While automated processes reduce direct contact with the seafood, it may also lead to increased bioaerosol production. In some cases, processing of the seafood is performed on board the shipping vessel which is characterised by confined spaces and inadequate ventilation systems.<sup>73</sup> The generated bioaerosols contains muscle, exoskeleton and visceral contents as well as various allergenic proteins. Other non-seafood components, which may also cause respiratory diseases are bacterial toxins (endotoxins), chemical additives such as sodium metabisulphites and spices such as garlic and paprika.

Various shellfish species are of considerable commercial importance, particularly king crab, snow crab and black tiger prawn which are processed on

a large scale. Several studies have been conducted analysing the airborne exposure of crustacean allergens in various processing activities.<sup>38, 73-79</sup> A recent study by Abdel Rahman *et al* has demonstrated the presence of major shrimp allergens in the bioaerosols generated in a processing facility particularly in the butchering section.<sup>80</sup> Processing procedures can vary from filleting, freezing, drying, cooking and high pressure techniques.<sup>81</sup> Specific activities that are known to cause excessive bioaerosol generation are butchering, meat grinding, degutting, boiling, degilling and cleaning of processing lines or storage tanks with high pressure water hoses. In addition, there has been recent evidence that high temperature and high pressure processing may affect the nature, dose and allergenicity of food.<sup>81, 82</sup>

Currently, there is a lack of standardised assays, which can detect and quantify the allergen concentrations in the breathing zones of workers stationed at different working activities. Better characterisation of the allergen repertoire in commonly consumed shellfish species along with the development of standardised approaches to identify and quantify allergens is of utmost importance for improved management of occupational allergies.

## 1.6 Food allergens

Allergenic proteins are present in different food sources in varying concentrations. Although several studies have been conducted previously to investigate the features that confer allergenic properties to specific proteins, there is still a lack of concrete methods to distinguish allergenic from non-allergenic proteins. More than 1200 allergenic proteins have been identified and sequenced from various sources and this number is steadily increasing. However, this constitutes only a fraction of the number of proteins that our immune system usually encounters.<sup>83</sup>

A protein is identified as an allergen when it causes a specific IgE response in at least 5 individuals.<sup>84</sup> The nomenclature and registration of newly identified allergens is developed and maintained by the World Health Organization/International Union of Immunological Societies Allergen Nomenclature Subcommittee ([www.allergen.org](http://www.allergen.org)).<sup>85</sup> Allergens are named by the first three letters of the genus, a single letter for the species followed by a number denoting the priority of allergen discovery.<sup>83</sup> Although this nomenclature is useful in locating the source of the allergen and its importance, it does not provide details of the intrinsic function of the allergenic protein or its identity. The identification of common functional and structural properties of allergenic proteins is essential for the basic understanding of allergic sensitisation and development of therapeutic strategies for the management of allergies.<sup>86</sup>

### 1.6.1 General characteristics of allergenic proteins

In the past few years, several studies have defined three important features that might singly or collectively render a protein allergenic. These are 1) surface features, 2) glycosylation and 3) protease activity. The most powerful property of allergens is its ability to initiate a T<sub>H</sub>2 response by targeting the innate defence of the body, mainly dendritic cells.<sup>86</sup> The elucidation of the primary structure or the amino acid sequence of the allergen helps in predicting the molecular weight, isoelectric point, hydrophobicity and stability using various bioinformatic approaches. Most of the identified allergens are low molecular weight proteins ranging from 10 to 70 kDa in size with low hydrophobicity and

high stability.<sup>85</sup> In addition, it has also been proposed that a common characteristic of allergenic proteins is the lack of bacterial homologues.<sup>87</sup>

### 1.6.2 Structural properties of allergenic proteins

Although more than a 1000 allergens have been identified and characterised, only 200 allergens have their tertiary structure elucidated in the RCSB protein Data Bank.<sup>85</sup> The secondary and tertiary structure of an allergenic protein is decoded using protein X-ray crystallography or NMR techniques. Most allergens can be grouped into 4 structural categories according to the protein folds. 1) antiparallel  $\beta$ -strands, 2) antiparallel  $\beta$ -strands with one or more  $\alpha$ -helices, 3)  $\alpha$ - and  $\beta$ - structures not closely associated; and 4)  $\alpha$ -helical structures. The allergens currently characterised, fall into one of these categories. However, these criteria alone are not enough to distinguish between allergenic and non-allergenic proteins.<sup>88</sup> The elucidated 3D structure of allergens or their homology models allowed for the possible explanation of cross-reactivity between homologues from different sources such as birch and apple.<sup>83</sup>

### 1.6.3 Allergen families and source of food allergens

Ingested allergens are present in various sources; however food sources such as peanuts, shellfish, fish, egg, milk, tree nuts, wheat and soy are responsible for the majority of allergic sensitisation. More importantly, more than one allergenic protein is present in each food source. For e.g. in peanuts (*arachis hypogaea*) thirteen allergenic proteins have been identified ([www.allergen.org](http://www.allergen.org)). Allergens to which more than 50% of the patients demonstrate reactivity are termed as major allergens.

A study conducted by Radauer *et al* in 2008 concluded that allergens belong to only selected protein families with limited number of biochemical functions.<sup>89</sup> Allergen families can be broadly classified based on, a) sources; animal, plant, fungi or bacterial origin, and b) route of exposure; ingestion, inhalation, contact, iatrogenic, sting/bite or autoallergen. The major allergen families for food allergens (through ingestion) were the prolamin superfamily, cupin family, EF hand domain, tropomyosin, profilin and *bet v 1* related proteins. Out of the 71

allergen families responsible for allergies through ingestion, these 6 families constitute 55% of the total allergens currently identified.

#### 1.6.4 Allergen surface properties

An allergic reaction is initiated when IgE antibodies on the surface of basophils and mast cells are cross-linked subsequent of binding to the allergenic protein. IgE antibodies bind to specific regions on the surface of these allergens. These regions are known as IgE epitopes. IgE epitopes constitutes usually of short amino acid sequences of 10-15 amino acids. Since the epitopes form a part of the molecular surface, the individual amino acids may originate from the same or different regions of the primary structure. These IgE epitopes are called sequential or conformational epitopes. Sequential epitope contain a linear section of the amino acid sequence of the allergen, whereas conformational or discontinuous epitopes constitutes of amino acids from different regions of the protein. The size and shape of conformational epitopes depend on the protein folds and charge of the allergen. Several studies have attempted to map the epitope regions of various food allergens, since the identity of IgE epitopes provide a glimpse into the cross-reactivity and stability of the allergen.<sup>65, 90-100</sup> Conformational epitopes may be prone to heat degradation and enzyme digestion as compared to linear or sequential epitopes.

An important feature of allergenic proteins, which may affect its IgE reactivity is the formation of homo-dimers or oligomers. Monomeric allergens are capable of triggering IgE cross-linking on the FcεRI receptors if the immune system has developed IgE antibodies targeted at non-overlapping epitopes on the allergenic protein.<sup>101</sup> However, in homo-dimeric allergens, the probability is higher for identical IgE antibodies to cross-link on the cell surface and trigger degranulation.<sup>102</sup> A study on birch pollen allergen has demonstrated the increase in allergenicity of Bet v 1 due to allergen oligomerisation.<sup>103</sup> However, such studies have not been performed for shellfish allergens.

#### 1.6.5 Shellfish allergens

Allergens in shellfish are mainly present in the edible portion of the animal. Over the past 20 years, shellfish allergens, particularly in crustaceans have

been identified and sequenced. The first such study was conducted in 1993 by Shanti et al in which the allergens SA-I and SA-II were identified as IgE binding proteins in *Penaeus indicus* (Indian white shrimp) bearing 86% amino acid identity with *Drosophila melanogaster* tropomyosin.<sup>104</sup> This was later identified to be tropomyosin, the major allergen found in crustaceans and molluscs.

Tropomyosin belongs to a family of highly conserved proteins with multiple isoforms found in muscle and non-muscle tissue of both vertebrate and invertebrate animals.<sup>105</sup> Tropomyosin exists as a complex with troponin and is involved in muscle contractile function by interacting with actin and myosin.<sup>106-109</sup> It is present in muscle (skeletal, cardiac and smooth), brain, platelets, fibroblasts, and many other non-muscle cells. In physiological state, tropomyosin exists as a highly stable  $\alpha$ -helical coiled coil homo-dimeric protein. Depending on alternate splicing mechanisms, different isoforms of tropomyosin are generated, which differ structurally and functionally. These are required for the regulation of contractility in different cell types.<sup>105</sup> Although these isoforms present in different tissues are homologous, structural differences do exist. Based on amino acid sequence differences, 12 different tropomyosin isoforms have been identified in rats.<sup>105</sup> In crustacean species, the fast twitch and the slow twitch isoforms were identified in the tail muscles and the pincer muscles, respectively.<sup>110</sup>

In muscle, two parallel alpha helical tropomyosin molecules are wound around each other forming a coiled-coil structure.<sup>106</sup> This form of secondary and tertiary structure imparts structural stability to tropomyosin and it is able to withstand heat- and high-pressure processing and retain its IgE binding capacity.

Over the years, several studies have identified tropomyosin to be the major allergen in various shellfish species. According to the Allfam database, tropomyosin is the fourth largest allergen family consisting of 47 tropomyosins identified in various food sources.<sup>89</sup> However, there is a lack of extensive studies investigating the effects of heat processing, cross-reactivity and altered IgE reactivity of this major allergen.

Other minor allergens have been identified in shellfish such as arginine kinase, myosin light chain and sarcoplasmic calcium binding protein.<sup>18, 111-118</sup> A few

studies have attempted to investigate the IgE binding to the identified shellfish allergens in various allergic cohorts. However, there is a lack of knowledge regarding the relevance of IgE binding to these allergens in various allergic populations and its relation to the severity of allergic symptoms. Nonetheless, identification and characterisation of shellfish allergens in various consumed species are essential for the development of improved diagnostic systems since the consumption of different species and subsequent exposure and sensitisation vary according to the geographical location.

### **1.6.6 Immunological allergen cross-reactivity**

One of the important features of major shellfish allergens is the phenomena of IgE antibody cross-reactivity. Tropomyosin is a highly conserved protein among various invertebrate species and demonstrates a high amino acid sequence identity. Because of this, IgE antibodies raised against tropomyosin from a certain species may bind to and trigger an allergic reaction upon exposure to tropomyosin from a different source. This immunological cross-reactivity may be responsible for cross-sensitisation and allergic reaction to house dust mites and insects among shellfish allergic patients.<sup>119-122</sup> A simple amino acid sequence alignment and comparison of the allergen sequences may be able to predict the level of IgE cross-reactivity. However, an in-depth investigation into the conservation or relevance of specific IgE epitopes among various tropomyosins is currently lacking. This information is essential for understanding the molecular basis of IgE cross-reactivity among various invertebrate species.

### **1.6.7 Effects of various food-processing methods on allergen reactivity**

Foods may undergo various processing phases for the purpose of preservation from microbes, modification to suit the end use (such as texture, taste or colour) and the improvement of digestibility. These processes can significantly alter the physicochemical and structural properties of the allergenic proteins thereby increasing or attenuating their allergenicity.<sup>123, 124</sup> Moreover, food processing can modulate the digestibility of allergenic proteins which may subsequently affect its presentation to intestinal immune cells.<sup>125</sup> Any process that modifies the allergen protein structure can be expected to alter its ability to be

recognised by antibodies.<sup>126</sup> In addition, such alterations in the protein structure can affect its detection using immunochemical methods such as ELISA and may lead to false negative results by failing to discriminate between the modified and un-modified allergens.<sup>127, 128</sup>

Food processing methods include mechanical processes, separation, biochemical processes, thermal processes, high pressure treatment, electric field treatment, and irradiation. Broadly, these methods can be categorised into thermal and non-thermal processes. Food processing methods may enhance, reduce or eliminate the allergenic potential of food. This is in turn affected by the factors such as heat, pH, moisture, pressure, concentration of proteins, etc.<sup>129</sup>

Thermal processing includes dry heat or moist heat treatment. In general, heat treatment reduces the IgE reactivity to the allergenic protein through unfolding mechanisms.<sup>130</sup> Such structural changes may disrupt the conformational epitopes resulting in the loss of allergic reactivity; for e.g. allergens *Mal d 1* and *Pru av 1* from apple and cherry, respectively.<sup>131, 132</sup> Apart from the direct effects of heating on the allergen structure, the food matrix also plays a role in the modification of allergenic activity. Therefore the presence of compounds such as fats and sugars are of importance.<sup>126</sup> One commonly occurring phenomena during thermal treatment is the Maillard reaction. This reaction occurs between the free amino acid residues of the proteins and aldehyde or ketone groups of naturally occurring sugars resulting in glycation of the amino acid residues.<sup>124</sup> The formation of such products is responsible for the aromas and flavours associated with many cooked foods.

The Maillard reaction products have two major effects on the allergen properties. Firstly, the glycation may result in resistance to digestion, thereby altering the presentation to intestinal immune cells and secondly, the modified allergens may elicit enhanced IgE reactivity as compared to the natural allergens. For example, the Maillard modifications of peanut allergens *Ara h 1* and *Ara h 2* form aggregates which bind to IgE more effectively and are also resistant to gastric digestion.<sup>133</sup> The type of heat treatment may also affect the allergen modification as demonstrated by increased IgE binding to *Ara h 1, 2*

and 3 from roasted peanuts as compared to boiled peanuts.<sup>134</sup> In another study, it was demonstrated that the Maillard modification of the allergen ovalbumin resulted in an increased uptake by dendritic cells.<sup>135</sup>

Novel processing techniques such as high-pressure processing and pulsed electrical field for sterilisation of food products are being increasingly used to reduce the negative effects of thermal treatment such as off-flavour or loss in texture.<sup>123</sup> In a recent study, pulsed electrical field treatment had no effect on the tested allergens, but high pressure and elevated temperatures lead to structural changes in apple allergens.<sup>136</sup> In a separate study on milk allergens, it was demonstrated that high-intensity ultrasound treatment did alter the IgE binding properties of the major whey allergens.<sup>137</sup>

In general, the conditions of processing, allergen composition, food matrix, allergen protein structure, presence of linear or conformational epitopes on the allergen and inherent stability can all influence the immune responses to modified or natural allergens.<sup>126</sup> However, the effect of food processing on the immunological reactivity or digestibility is not yet entirely predictable, though some general rule exists.<sup>123, 138</sup>

#### **1.6.7.1 Effects of food processing on shellfish allergens**

Several commercially important shellfish species undergo food processing steps, such as preservation and sterilisation, prior to consumption. In majority of the cases, thermal processing techniques are used for this purpose. However, the effects of heat processing on the IgE reactivity and structural stability of shellfish allergens have not been investigated in detail, with only a few studies conducted in the past ten years.

A study conducted in 2010 by Liu *et al* analysed the effects of boiling shrimps on the IgE binding of the major allergen tropomyosin. It was demonstrated that the IgE binding to tropomyosin was enhanced after heat treatment.<sup>139</sup> Another study in 2010 investigated the effects of different processing methods on the digestibility of crab tropomyosin.<sup>140</sup> Three different processing methods; boiling, ultrasound and high pressure steaming were used prior to simulated digestion of the allergenic protein. It was demonstrated that ultrasound and high pressure

processing promoted the digestion of tropomyosin, subsequently reducing the IgE binding properties *in vitro*. However, the processing methods employed in this study were based on lab scale equipments and did not represent industrial grade food processing. A study in 2005 investigated the effects of Maillard reaction on the IgE binding properties of scallop tropomyosin.<sup>141</sup> Enhanced IgE binding was observed to the Maillard products of tropomyosin. In contrast, another study in 2006 demonstrated reduced IgE binding to the Maillard products of squid tropomyosin.<sup>142</sup> In both these studies, tropomyosin was analysed from the crude mollusc extracts. Investigation of the Maillard reaction using recombinant purified shellfish allergen would allow for detailed analysis of the structural changes and altered IgE reactivity.

In terms of non-thermal food processing methods, a study in 2011 by Shriver *et al* demonstrated that pulsed ultraviolet light treatment was able to reduce the reactivity of shrimp tropomyosin with decreased IgE binding to the shrimp extract.<sup>143</sup>

Recent advances in the field of proteomics and functional cellular assays have made it possible to analyse the effects of thermal processing of shellfish on the IgE reactivity of allergens in more detail.<sup>144</sup> Identification of the whole allergen repertoire in the different shellfish species and analysis of the effects of thermal processing on the stability and IgE reactivity using recombinant allergens is essential in the development of improved allergen diagnostic approaches.

## 1.7 Diagnosis of shellfish allergy

Diagnostic methods of establishing a true seafood allergy include various *in-vivo* and *in-vitro* tests to demonstrate the presence of specific IgE antibodies.<sup>145</sup> Due to the possible unavailability of the exact species using SPT and blood IgE assays, positive and negative test results should be supported by a clinical history of the patient and/or oral challenge where possible. An accurate evaluation of shellfish allergy using the best *in-vivo* and *in-vitro* tests will result in a less restricted dietary curtailment than is currently recommended. The common methods and approaches are summarised in Figure 1.3.

### 1.7.1 History

A precise and detailed history is very important to gain information regarding the seafood species under suspicion, nature of the symptoms and the atopic status of the patient. In addition, the identification of the implicated seafood species using specific diagnostic procedures is of importance, particularly if mislabelling of a seafood product is a possibility. An atypical clinical history or an inconsistent history always suggests a non-atopic aetiology, such as contamination with toxins or parasites or intolerance reaction to seafood.

### 1.7.2 Skin tests

The use of commercial skin prick tests is often considered and two different providers of shellfish skin prick test (SPT) solutions in Australia are highlighted as examples in Table 1.2. In some cases the species name is not provided for single allergens as well as for mixed tests. In addition much fewer SPT solutions seem to be available for the mollusc group as compared to the crustacean group. If specific extracts are not available, so called in-house prepared SPT extracts can be utilised, if they are confirmed to be safe for testing (no toxins) and contain the appropriate allergens.<sup>72</sup> Despite the drawbacks of possible false positive/negative results obtained with skin prick testing, if performed properly and with the appropriate shellfish extracts, it is a quick and sensitive test.<sup>146</sup>

**Table 1.2:** Some commercial Skin Prick tests available in Australia for crustacean and mollusc antigens. Bold letters/numbers indicate the test code.

Commercial Skin Prick tests		Crustacean specific (Test code)	Source	Mollusc specific (Test code)	Source of the protein extract
ALK-Abello	Soluprick (Spain)	<ul style="list-style-type: none"> <li>Crab (<i>Cancer pagurus</i>) (6.9)</li> <li>Shrimp (<i>Pandalus borealis</i>) (6.89)</li> </ul>	Fresh meat	None	?
	Allergenic Extract (USA)	<ul style="list-style-type: none"> <li>Crab (<i>Paralithodes camtschatica</i>) (CRAB)</li> <li>Lobster (<i>Panulirus spp.</i>) (LOBS)</li> <li>Shrimp (<i>Penaeus spp.</i>) (SHRI)</li> <li>Shellfish, Mixed (Crab, Shrimp, Lobster, Oyster) (MISH)</li> <li>Shellfish Mix 4 (Crab, Clam, Lobster, Shrimp) (SHM4)</li> </ul>	Fresh meat	<ul style="list-style-type: none"> <li>Oyster (<i>Ostrea spp.</i>) (OYST)</li> </ul>	Fresh meat
Stallergenes		<ul style="list-style-type: none"> <li>Shrimp (120)</li> <li>Spiny Lobster (131)</li> </ul>	?	<ul style="list-style-type: none"> <li>Mussel (139)</li> <li>Oyster(131)</li> </ul>	?

? – Source is unknown

### 1.7.3 Blood IgE tests

A precise and reliable *in-vitro* assay to quantify the amount of allergen specific IgE antibodies is a valuable tool to support the clinician in confirming or refuting an allergic reaction to seafood, prescribing medication and following up treatment and predicting disease development. Detecting and quantifying IgE antibodies, however, is considerably more complicated than performing many other immunoassays.

There are a number of complicating factors to be considered:

- The concentration of IgE antibodies in blood is extremely low (0.05% as compared to 75% for the IgG isotype), even in highly sensitised individuals.
- Each allergen source contains a large number of different allergenic components (mostly proteins). The assay must therefore be sensitive enough to capture antibodies to all relevant allergens, even if these are present only in very minute amounts.
- The assay must have high enough capacity to bind all IgE antibodies to an allergen in competition with other antibodies with the same specificity from other immunoglobulin classes present in higher concentrations (e.g. IgG)
- To achieve a precise and reproducible test system, total control of the allergen source material is necessary, both in content and in allergenic activity, thus reassuring reproducibility when comparing different patients.

There are several commercial tests available to quantify specific IgE antibodies; however, the most prominent system is the ImmunoCAP (Thermo), which has been used as a model system to demonstrate the gaps and needs in the context of seafood allergy diagnosis. The ImmunoCAP test (previous known as CAP-RAST) is an *in-vitro* diagnostic test to measure the amount of specific IgE antibodies to a given allergen.<sup>76, 145, 147</sup> The accuracy of this assay is dependent on the selection of the correct seafood species and is restricted to the panel of commercially available species.

Table 1.3 and Table 1.4 list the names of some important crustacean and mollusc species in Australia as well as the currently available commercial ImmunoCAP tests. The repertoire of species available for testing is limited,

particularly for mollusc species. A common problem to the clinician is the correct choice of test as the common names often cause confusion. One example is the analysis of allergic reactions to 'Crayfish'. The correct application of this species name is for a freshwater crustacean species; for e.g. Crawfish (USA), Red claw, Yabby (Australia). However, commonly Rock Lobster is also called 'Crayfish' adding to the confusion, as the former is actually a Spiny Lobster. The most appropriate test species would be in this case the ImmunoCAP for Spiny Lobster/Langoustine, Rf304 (Table 1.3). Due to the vast amount of different shrimp/prawn species worldwide available an improved ImmunoCAP has been developed (f24) which includes four different species (Table 1.3). Many patients with seafood allergy have simultaneous sensitivity to other seafood species, but some patients are truly mono-sensitive to a particular species. In addition the use of allergens derived from raw or heat treated sources must be considered, as differential allergic responses have been documented.<sup>148, 149</sup> Some of the allergens used for example in the ImmunoCAP's are identified as being derived from heated extracts (e.g crab and some prawns), whereas this information is not known for the majority of shellfish and mollusc allergens.

In general a negative test result excludes sensitisation to these constituents whereas a positive result could be followed up with a specific ImmunoCAP for the individual component allergen. However a positive history of shellfish allergy and negative ImmunoCAP result needs further investigation and should be followed up by additional investigations.

**Table 1.3:** Assays to quantify specific serum IgE. Commonly consumed allergenic crustacean species including their scientific names are listed and currently available ImmunoCAP tests and their crustacean species utilised. Bold numbers indicate test codes.

Group		Commonly consumed species	Species used for ImmunoCAP® tests (Test code)	Source of the protein extract	
Crustacea	Shrimps, prawns	<ul style="list-style-type: none"> <li>Black tiger prawn (<i>Penaeus monodon</i>)</li> <li>Vannamei prawn (<i>Litopenaeus vannamei</i>)</li> <li>Brown shrimp (<i>Penaeus aztecus</i>)</li> </ul>	Shrimp mix ( <b>f24</b> ) <ol style="list-style-type: none"> <li>Black tiger prawn (<i>Penaeus monodon</i>),</li> <li>Northern shrimp (<i>Pandalus borealis</i>),</li> <li>Velvet prawn (<i>Metapneaeopsis barbata</i>),</li> <li>Shiba shrimp (<i>Metapenaeus joyneri</i>)</li> </ol>	Raw or boiled frozen meat	
	Crabs	<ul style="list-style-type: none"> <li>Mud crab (<i>Scylla serrata</i>)</li> <li>Snow crab (<i>Chionoecetes opilio</i>)</li> <li>King crab (<i>Paralithodes camtschaticus</i>)</li> </ul>	Crab ( <b>f23</b> ) <ul style="list-style-type: none"> <li>Edible crab (<i>Cancer pagurus</i>)</li> </ul>	Boiled meat	
	Lobsters		<ul style="list-style-type: none"> <li>Southern Rock lobster (<i>Jasus edwardsii</i>)</li> </ul>	Lobster ( <b>f80</b> ) <ul style="list-style-type: none"> <li>European lobster (<i>Homarus gammarus</i>)</li> </ul>	?
			<ul style="list-style-type: none"> <li>Spiny lobster (<i>Panulirus stimpsoni</i>)</li> </ul>	Spiny lobster ( <b>f304</b> ) <ul style="list-style-type: none"> <li>Common spiny lobster (<i>Palinurus vulgaris</i>)</li> </ul>	?
			<ul style="list-style-type: none"> <li>Crayfish, yabby (EU; Australia) (<i>Astacus spp</i>, <i>Cherax spp</i>)</li> <li>Crawfish (USA) (<i>Procambarus spp</i>)</li> </ul>	Crayfish ( <b>f320</b> ) <ul style="list-style-type: none"> <li>Louisiana crayfish (<i>Astacus astacus</i>)</li> </ul>	Boiled meat and shell
	Crustacean allergen component	Shrimp ( <i>Penaeus aztecus</i> )	Tropomyosin, rPen a1 ( <b>f351</b> )	Recombinant purified allergen	

**Table 1.4:** Assays to quantify specific serum IgE. Commercially available ImmunoCAP tests and mollusc species utilised are listed. Bold numbers indicate the specific test

Group		Commonly consumed species	Species used for ImmunoCAP® tests (Test code)	Source of the protein extract
Mollusca	Gastropoda	• Abalone ( <i>Haliotis midae</i> )	• Abalone ( <i>Haliotis spp</i> ) ( <b>f346</b> )	Whole meat
		• Snail ( <i>Helix aspersa</i> )	• Snail ( <i>Helix aspersa</i> ) ( <b>f314</b> )	Whole meat
		• Periwinkle ( <i>Oxystele spp.</i> )		
	Bivalvia	• Black mussel ( <i>Choromytilus meridionalis</i> )	• Blue Mussel ( <i>Mytilus edulis</i> ) ( <b>f37</b> )	Canned meat
		• Red oyster ( <i>Ostrea atherstonei</i> )	• Oyster ( <i>Ostrea edulis</i> ) ( <b>f290</b> )	Fresh meat
		• White mussel ( <i>Donax serra</i> )	• Clam ( <i>Ruditapes spp.</i> ) ( <b>f207</b> )	Fresh frozen meat
			• Scallop ( <i>Pecten spp.</i> ) ( <b>f338</b> )	?
	Cephalopoda	• White squid ( <i>Loligo vulgaris reynaudii</i> )	• Squid ( <i>Loligo edulis, Loligo vulgaris</i> ) ( <b>f258</b> )	?
		• Red squid ( <i>Todarodes angolensis</i> )	• Pacific squid ( <i>Todarodes pacificus</i> ) ( <b>f58</b> )	?
		• Octopus ( <i>Octopus vulgaris</i> )	• Octopus ( <i>Octopus vulgaris</i> ) ( <b>f59</b> )	Fresh frozen meat

codes.

“?” – Source is not known

#### 1.7.4 Allergen Microarray technology

A novel antibody detection system, the allergen-microarray has emerged as a promising approach to high-throughput large-scale profiling of allergen interactions for simultaneous monitoring of IgE and IgG antibodies directed against a variety of allergy-eliciting molecules.<sup>150-153</sup> A number of allergens are spotted onto a solid phase (e.g., modified glass slides or nitrocellulose membranes) and subsequently used to bind antibodies from the serum of allergic patients. Detection of allergen-specific antibody binding is accomplished by the addition of specific secondary antibodies that carry an appropriate label for the quantification using laser technology and are quantified in terms of g/l or IU/ml. The major benefit of this technology lies in its ability to screen for several hundred allergen molecules simultaneously while employing only minute amounts of the patients' serum (usually 20  $\mu$ L). The capturing agents that can be used are either crude or partially purified allergen extracts, highly purified recombinant or natural allergenic components. Subsequently this will result in an optimal profiling of the patients IgE response (in one analytical step), identifying major and minor allergens, pan-allergens as well as possible cross-reactive carbohydrate determinants (CCDs).

#### 1.7.5 Immunoblot (Western blot)

Sometimes patients present with a clear history of allergic sensitisation to shellfish but commercially available assays do not detect elevated specific antibodies. In this case when sensitisation to an unknown allergen source is suspected, Western-blotting (immunoblotting) can be conducted. Protein extracts of the offending allergen source are separated by gel-electrophoresis (in an electrical field) according to molecular size; the allergens are then transferred to a membrane (blotting) and detected with serum IgE antibodies from sensitized patients.<sup>154, 155</sup> This method can be very sensitive and allows the direct identification of specific allergens (major and minor) for individual patients. In addition this method allows comparison of IgE reactivity to raw and heated allergen sources, which might be of clinical importance.

### 1.7.6 Basophil stimulation assay

The results of immunoassays for the detection of specific IgE antibodies are potentially susceptible to the presence of IgG antibodies of the same specificity, can vary between the different assay systems and often do not correlate with data obtained by skin testing. With these issues in mind more sensitive and specific functional *in-vitro* tests have been developed to investigate the cause of allergic reactions. These functional *in-vitro* assays focus on basophil-mediator release assays such as histamine and leukotriene release tests and recently focus on the utilisation of flow cytometry.<sup>156</sup>

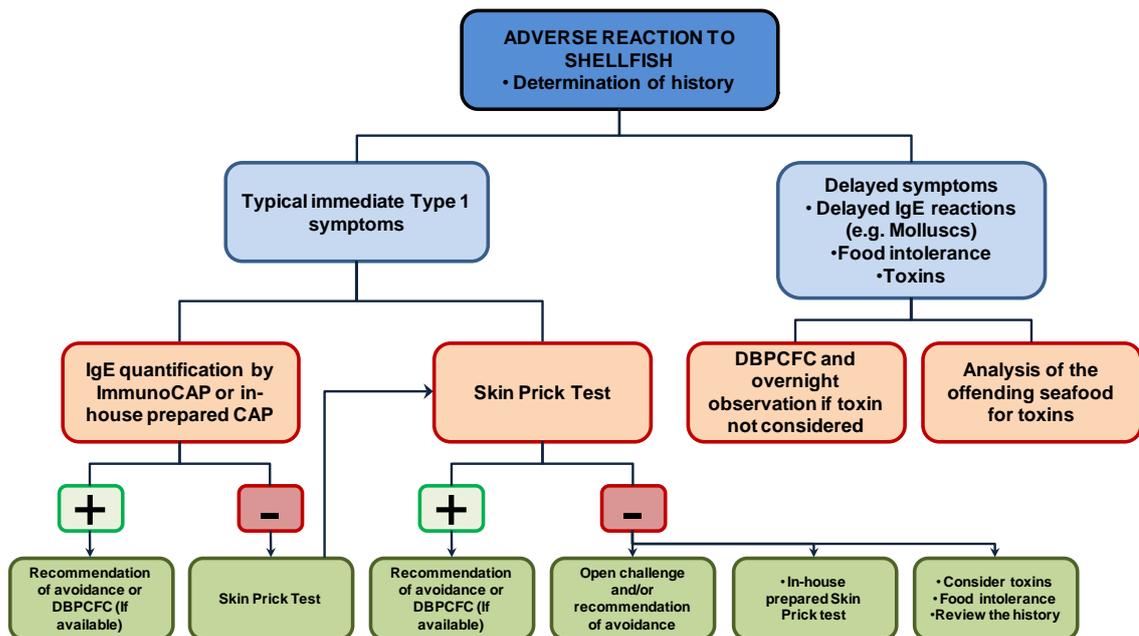
Human basophils express a variety of cytokine receptors including receptors for the IgE antibody (FcεRI). The basophils can be activated by cross-linking of these IgE binding receptors with the specific allergen resulting in the release of vesicles containing mediators such as histamine and leukotrienes. Recently new specific marker proteins, known as CD63 and CD203c were discovered on basophils and observed that these proteins were associated with secreted granules and up-regulated concomitantly with basophilic degranulation. These two marker proteins are now used to demonstrate activation of basophils using flow cytometry. This assay is relatively fast and needs about 2-5 ml of whole blood. Protein allergens can easily be tested, however, healthy control subjects have to be included and assessed for each allergen.<sup>157, 158</sup>

### 1.7.7 Double-Blind Placebo-Controlled Food Challenge (DBPCFC)

The “gold standard” for diagnosis of food allergy and for the identification of the offending agent is the DBPCFC. Various studies indicate a range of minimal shellfish concentrations to elicit clinical reactions. Wu and Williams reported that fatal anaphylaxis occurred after ingestion of three snails.<sup>146</sup> A different study using DBPCFC reported the accumulated amount of as little as 120 mg of dried snail caused a significant decrease in FEV1 (Forced expiration volume).<sup>159</sup> For crustacean Bernstein *et al* reported that patients in a DBPCFC reacted to 14 gram of shrimp.<sup>160</sup> Similar results were confirmed by Daul *et al* which reported that the equivalent dose of about four medium-sized shrimps (16 gram) caused reactions in DBPCFC.<sup>161</sup> However, this technique does not distinguish between allergic (IgE mediated) and non-allergic hypersensitivity

involving different antibody types, cellular immune mechanisms and reactions based on intolerance or toxins. However, performing oral food challenges can improve the quality of life, particularly when the results are favourable.<sup>162</sup>

In summary, various diagnostic tests are used only in support to medical history and epidemiology of the food allergy. While allergists avoid invasive allergy testing, sIgE and total IgE quantification from *in vitro* testing only predicts the severity of clinical symptoms. However, the next generation sIgE quantification involving specific allergen “components” within foods, often termed as Component Resolved Diagnosis (CRD) may provide an improved or refined testing platform better able to predict the allergic sensitisation in combination with other parameters.



**Figure 1.3:** Diagnostic algorithm for shellfish allergy (DBPCFC – double-blind, placebo-controlled food challenge).

## 1.8 Allergen detection and quantification

The detection and quantification of allergens is an important responsibility in the food processing industry, where allergenic proteins present in trace amounts may cause accidental exposure and clinical reaction in affected individuals. Allergens may be unintentionally introduced in food products due to the sharing of production lines or shared air ventilation. Moreover, certain shellfish products are commonly used as flavouring agents in packed food products thus introducing allergens in trace amounts.

To avoid accidental exposure and reaction, food labelling practices and regulations are implemented by specific legislations declaring the allergen contents in the given food product. The basis for labelling in most countries is provided by the International Codex Alimentarius Commission.<sup>163</sup> 14 different food groups are required for allergen labelling in the European Union as compared to 5 allergens in Japan.<sup>164</sup> Moreover, The European Commission food labelling law requires crustaceans and molluscs to be declared separately.<sup>165</sup>

The identification and detection of food allergens remains a challenging issue. Such analysis is complicated by the complex food matrices, presence of multiple allergens, trace amounts of allergens, etc. There is a lack of standardised analytical methods for the detection of shellfish allergens in food including antibody-based methods and mass spectrometric approaches.<sup>144</sup>

Previous studies in the past 10 years have attempted to develop analytical methods for the sensitive detection of shellfish allergens. Antibody-based immunoassays have been frequently used for allergen detection because of its ease of use, sensitivity and low assay variability. Monoclonal and polyclonal antibody-based immunoassays have been developed for the detection of the shellfish allergen, tropomyosin.<sup>166-169</sup> However, most of these assays differ in their sensitivity and specificity to various shellfish species. A study in 2011 by Taguchi *et al*, used the polymerase chain reaction (PCR) technique for the detection of shrimp and crab genomic DNA as a means of detecting trace amounts of allergenic content.<sup>170</sup>

Recent advances in the use of next generation mass spectrometric approaches provide an opportunity to improve the sensitivity of analytical methods for allergen detection and overcome the drawbacks of ELISA based assays such as matrix interference.<sup>144</sup> Moreover, multiple allergens may be detected in a broad concentration range (up to five magnitudes). Currently, there is a lack of a robust antibody-based or chemical-based analytical tool for the sensitive detection of shellfish allergens.

## 1.9 Summary and Research Synopsis of the Thesis

In this Chapter, a detailed review on the current status of food allergy and diagnostic approaches was provided with insights into the prevalence of shellfish allergy, clinical manifestations of allergic reactions, effects of food processing on allergenic protein structure, and allergen detection.

The work presented in this PhD Thesis provides a comprehensive study on the identification and characterisation of major and novel allergens in commonly consumed shellfish species specific to the Asia-Pacific region. Particular emphasis was laid on the effects of thermal processing of shellfish on the IgE antibody reactivity of allergenic proteins and subsequent investigation of immunological cross-reactivity of the major allergens.

In **Chapter 2**, an extensive panel of shellfish allergen extracts was generated to analyse the effect of heat treatment on the detection of the major allergen, tropomyosin using a commercial monoclonal antibody. Black tiger prawn (*Penaeus monodon*) was investigated in further detail for the identification of the prawn allergen repertoire.

**Chapter 3** details the development and validation of novel mass spectrometric methods implemented for the identification and sequencing of tropomyosin and other shellfish allergens.

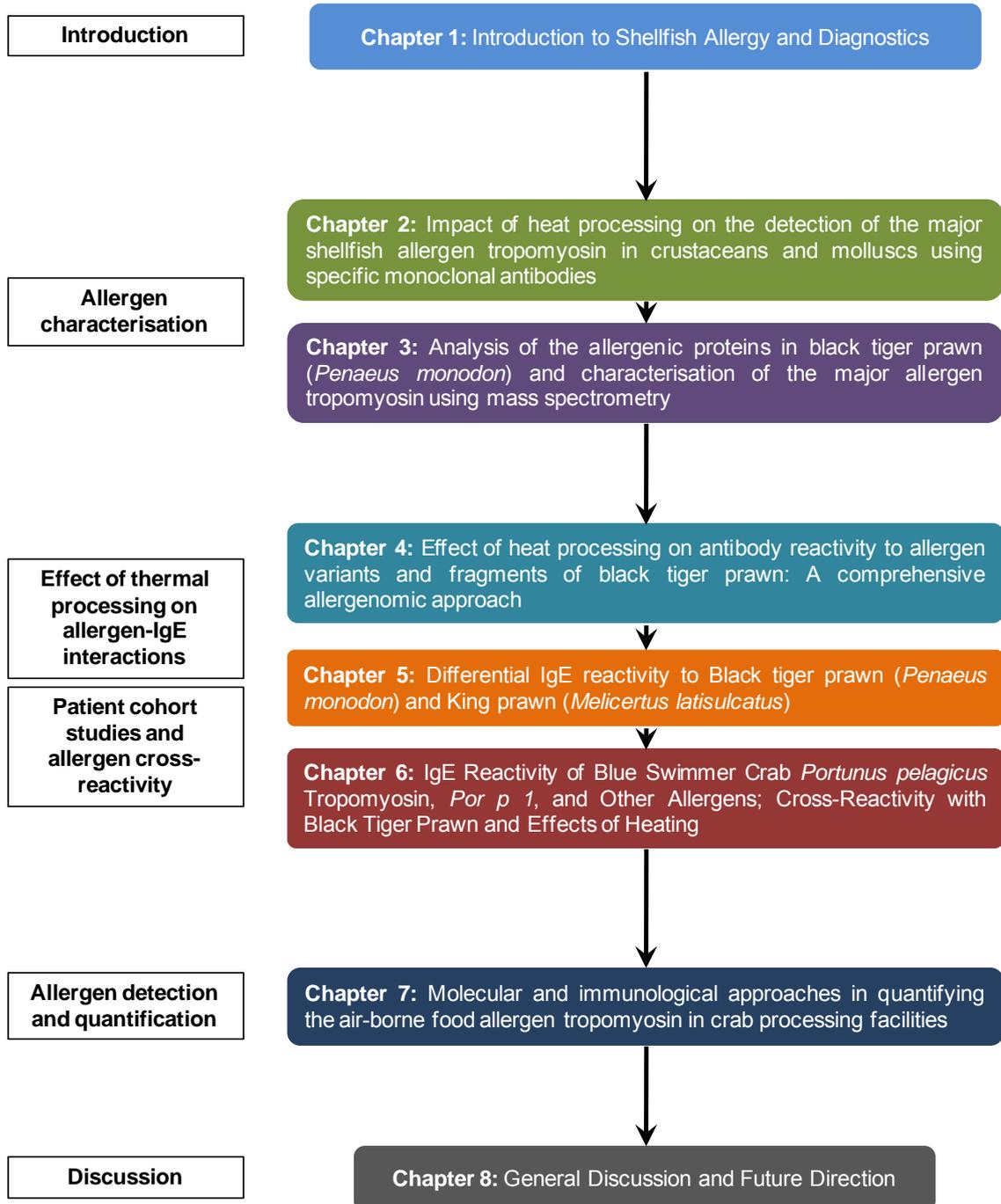
In **Chapter 4**, allergens in Black tiger prawns were characterised based on patient IgE binding and mass spectrometric identification. Differential IgE binding to untreated and heat-treated prawn allergens were investigated using patient serum IgE.

**Chapter 5** details the identification and characterisation of tropomyosin from a different prawn species important to Australia, King prawn (*Melicertus latisulcatus*). Furthermore, the differential IgE binding between Black tiger prawn (*Penaeus monodon*), *Pen m 1* and King prawn tropomyosin, *Mel I 1* were investigated. A detailed amino acid sequence analysis was conducted to investigate the molecular basis of IgE cross-reactivity.

**Chapter 6** details the identification and characterisation of tropomyosin from a different crustacean group of crabs; specifically the Blue swimmer crab tropomyosin, *Por p 1*. IgE binding and clinical cross-reactivity of *Pen m 1* and *Por p 1* in shellfish allergic patients were investigated using inhibition ELISA and basophil activation assay.

In the final **Chapter 7**, a sensitive immunoassay was developed and validated for the quantification of the aerosolised allergen tropomyosin in crab-processing factories. Using this sensitive assay, tropomyosin was quantified in the personal breathing zones of 80 crab processing workers, thus identifying high-risk activities.

The work presented in this thesis provides an important contribution towards the development of improved and sensitive allergy diagnostic platforms.



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## CHAPTER 2

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### IMPACT OF HEAT PROCESSING ON THE DETECTION OF THE MAJOR SHELLFISH ALLERGEN TROPOMYOSIN IN CRUSTACEANS AND MOLLUSCS USING SPECIFIC MONOCLONAL ANTIBODIES

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## 2.1 Introduction

Seafood plays an important role in human nutrition and health. The growing international trade in seafood species and products has added to the popularity and frequency of consumption of a variety of seafood products across many countries. This increased production and consumption of seafood have been accompanied by more frequent reporting of allergic health problems among consumers. Allergic reactions are manifested by gastrointestinal and dermatological symptoms as well as respiratory and anaphylactic reactions.<sup>1, 2</sup> The appearance of allergic symptoms results not only from ingestion of seafood, but can also be triggered from inhaling cooking vapours and handling shellfish.<sup>3-5</sup> Importantly, patients with shellfish allergy, similarly to those with peanut allergy, mostly remain clinically reactive throughout their lives and are at increased risk for wheezing illness and hyper-reactive airways at school age.<sup>2</sup>

The three most important seafood groupings causing allergic reactions include fish, crustaceans and molluscs. The latter two phyla of crustaceans and molluscs are generally referred to as 'shellfish' in the context of seafood consumption. The allergic response in sensitised consumers is mediated by serum IgE antibodies directed to specific allergens such as the major allergen tropomyosin, an abundant shellfish muscle protein.<sup>6</sup> The presence of this allergenic protein in processed food, even at very low concentrations, can cause severe reactions in sensitised consumers. Therefore the labelling of food products containing crustaceans has already become mandatory in many countries including the USA, Europe and Japan. Recently the European Union adapted guidelines to include molluscs as a separate food allergen, based on the limited cross-reactivity to crustacean allergens.<sup>7</sup> Several studies have shown the ability of tropomyosin to withstand heating or cooking procedures and elicit IgE antibody binding. Moreover, it has been shown that some type of heat-treatment can alter the digestibility of tropomyosin.<sup>8</sup> However, a detailed comparative study on the effects of heat-processing on the antibody binding and detection of tropomyosin from different shellfish species is still lacking.

Commercially available shellfish allergen detection kits usually make use of polyclonal rabbit anti-allergen antibodies. However their ability to differentiate

between the major allergens from crustaceans and molluscs is often not defined, and in the case of such polyvalent rabbit antibodies it is very difficult to achieve. In order to identify and characterise the major shellfish allergen tropomyosin, several monoclonal and polyclonal antibody based assays have been developed. However, the species-specificities and binding epitopes of these antibodies have not been studied in detail. In addition, there are no monoclonal antibodies available for the detection and quantification of tropomyosin from crustaceans and molluscs.

A monoclonal anti-insect tropomyosin antibody (MAC 141) from Abcam, Cambridge, USA is available commercially with binding specificity to invertebrate tropomyosin. This antibody was raised against the water bug insect (*Lethocerus indicus*) flight muscle tropomyosin. Given the high amino acid sequence conservation of tropomyosin among invertebrate species, the antibody binding to shellfish tropomyosin and cross-reactivity warranted further investigation. This was the first ever commercially available monoclonal antibody to be tested as a detection tool for shellfish tropomyosin.

## 2.2 Aims

The aims of this study were as follows

- 1) To evaluate the use of allergen-specific monoclonal antibodies for the detection of shellfish derived tropomyosin in a comprehensive range of crustacean and mollusc species.
- 2) To analyse the impact of heat-processing on antibody recognition towards tropomyosin for improved allergen detection in processed food.

## 2.3 Materials and Methods

### 2.3.1 Shellfish samples

Fresh or frozen specimens of 11 different crustacean and 7 mollusc species were acquired from local markets and distributors across Melbourne, Australia, as listed in Table 2.1.

**Table 2.1:** Common and scientific names of the eleven crustacean and seven mollusc species analysed in this study. The theoretical molecular weight and GenBank accession numbers of characterised tropomyosins are listed for each species if available

No	Shellfish Species		Theoretical MW (kDa)	Accession numbers (GenBank)		
	Common name	Scientific name				
Crustaceans	Prawn	black tiger prawn	<i>Penaeus monodon</i>	32.8	HM486525	
		king prawn	<i>Melicertus latisulcatus</i>	32.6	JX171685	
		vannamei prawn	<i>Litopenaeus vannamei</i>	32.8	EU410072	
		banana prawn	<i>Fenneropenaeus merguensis</i>	32.8	GU369817	
		green tiger prawn	<i>Penaeus semisulcatus</i>	-	-	
	Crab	blueswimmer crab	<i>Portunus pelagicus</i>	32.8	JX874982	
		sand crab	<i>Ovalipes australiensis</i>	-	-	
		snow crab	<i>Chionocetes opilio</i>	32.6	BAF47267	
	Lobster	slipper lobster	<i>Thenus orientalis</i>	32.0	KC291443	
		rock lobster	<i>Jasus edwardsii</i>	32.9	KC291442	
		yabby	<i>Cherax destructor</i>	32.0	KC291443	
Molluscs	Bivalve	green mussel	<i>Perna viridis</i>	32.7	AAG08988	
		blue mussel	<i>Mytilus edulis</i>	32.7	U40035	
		scallop	<i>Pecten fumatus</i>	-	-	
		oyster	<i>Crassostrea gigas</i>	33.0	BAH10152	
	Gastropod	sea snail	<i>Turbo cornutus</i>	32.7	AB444940	
		Cephalopod	octopus	<i>Octopus vulgaris</i>	32.8	BAE54433
			calamari (squid)	<i>Sepioteuthis lessoniana</i>	32.6	AB218914

The specimens were transported to the laboratory on ice and frozen at  $-20^{\circ}\text{C}$  until further use.

### **2.3.2 Preparation of protein extracts**

For the preparation of raw protein extract, the outer shell of the specimen was removed and the edible meat cut into small pieces. The abdominal or tail muscles were used from prawns, crabs and lobster specimens. For the bivalves, the shell was split open and the inner muscle parts used for extraction. 50 gm of the muscle mass was homogenised in 150 mL of phosphate buffered saline (PBS) for 10 minutes using an Ultra turrax blender (IKA, Staufen, Germany). This slurry was then agitated for 3 hours at  $4^{\circ}\text{C}$  followed by centrifugation at 14,000 rpm for 15 minutes. The supernatant was clarified through a glass fibre filter, followed by filtration through a  $0.45\ \mu\text{m}$  membrane filter (Millipore, Billerica, MA, USA) and stored at  $-80^{\circ}\text{C}$  until further use.

For the generation of heated protein extracts, a more natural way of heat treatment was utilised, instead of directly heating the raw extract, to mimic the way consumers are usually exposed to food allergens. The complete shellfish specimen, in its outer shell, was heated in liquid (PBS) at  $100^{\circ}\text{C}$  for 20 minutes. The outer shell was removed after cooling and the proteins from these muscle tissues were extracted using the same method as described for the raw extract.

### **2.3.3 Protein quantification**

The total protein content of each prepared extract was determined using the Quick Start Bradford Assay kit (BioRad, USA) following the manufacturer's instructions. Bovine serum albumin (BSA) was used as the protein standard.

### **2.3.4 SDS-PAGE analysis**

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) was performed to visualise the total protein repertoire in the prepared extracts

as described previously.<sup>9</sup> Twelve µg of protein extract was briefly heated in Laemmli buffer with dithiothreitol and loaded onto a 12% bis-acrylamide gel. Electrophoretic separation was performed at 170V until the tracker dye reached the base using a Mini-Protean Tetra Cell electrophoresis system (BioRad, Hercules, CA, USA). The separated proteins were visualised by staining with Commassie brilliant blue R250 (BioRad, Hercules, CA, USA).

### **2.3.5 Immunoblotting**

#### **2.3.5.1 Immunoblotting with monoclonal anti-tropomyosin antibody**

Four µg of the crustacean protein extract was resolved by SDS-PAGE as detailed above. The separated proteins were transferred to an activated Polyvinylidene fluoride (PVDF) membrane using the Semi-dry TransBlot Apparatus (BioRad, Hercules, CA, USA). After blocking with 5% (w/v) skim milk powder (SMP) in PBS-T, the membrane was subsequently incubated with monoclonal anti-insect tropomyosin antibody, mac-141 (Abcam, Cambridge, MA, USA) diluted 1:6000 in 1% SMP, PBS-T and rabbit anti-mouse IgG antibody conjugated with HRP (Sigma, St. Louis, MO, USA) diluted 1:50,000. After washing three times with PBS-T, the membrane was visualised using the enhanced chemiluminescent technique as reported previously.<sup>10, 11</sup> Briefly, the blots were incubated with chemiluminescent substrate (Sigma, St. Louis, MO, USA) and exposed to photographic film (GE Healthcare Biosciences, USA) to visualise the antibody binding protein bands.

#### **2.3.5.2 Patient sera IgE Immunoblotting**

To confirm the allergenicity of tropomyosin, IgE antibody reactivity was evaluated by immunoblotting using a pool of sera from patients with confirmed allergy to shellfish. IgE immunoblotting was performed as described previously.<sup>12</sup> Briefly, the proteins on the membrane were incubated with patient sera (1:10 in 1% SMP, PBS-T) and subsequently incubated with rabbit anti-human IgE polyclonal antibody (DAKO Corporation, Carpinteria, CA, USA) and goat anti-rabbit IgG labelled with HRP (Promega, USA). IgE antibody binding was visualised as described above. A healthy donor's serum was used as a

negative control. Ethics approval for this study was granted by James Cook University's Ethics committee (Project number H4313) in collaboration with The Alfred Hospital (Project number 192/07) and Monash University's Ethics Committees (MUHREC CF08/0225).

### 2.3.6 Purification of natural tropomyosin

Tropomyosin from black tiger prawn was purified through anion-exchange chromatography as described previously<sup>9</sup> using a Biologic LP fast protein liquid chromatography system (BioRad, Hercules, CA, USA). About 20 mg of the protein extract was diluted in starting buffer of 30mM acetate buffer, pH 5.5, loaded onto a Mini Macroprep High Q column (BioRad, Hercules, CA, USA) and tropomyosin eluted using an increasing NaCl concentration (0.4 M to 0.6 M) The collected tropomyosin fraction was further purified through a Sephadex G-50 gel filtration column (Sigma, MO, USA) using PBS as medium and subsequently concentrated using an Amicon spin column (Merck, USA) and stored at -80°C until further use.

### 2.3.7 Mass spectrometric identification of tropomyosin

The IgE and monoclonal antibody reactive prawn tropomyosin was excised from the SDS-PAGE gel for mass spectrometric analysis. The band was de-stained and digested with trypsin as previously reported.<sup>10, 13</sup> About 250 fmole of total protein was injected into a DIONEX Ulti-242 Mate3000 Nano LC System (Germering, Germany) and the tryptic peptides separated on a nanoflow analytical column (75  $\mu\text{m}$   $\times$  15 cm, C18 PepMap 100, 3  $\mu\text{m}$ , 100 A, (LC Packing, Sunnyvale, CA) at 180 nL/min using a gradient regime. The resultant tandem spectra were searched using the National Center for Biotechnology Information non-redundant database (NCBI nr) with the Matrix Science (Mascot) search engine (precursor and product ion mass tolerance set at 0.2 Da). Methionine oxidation was allowed as a variable modification and guanidinylation (K) as a fixed modification since the guanidination derivatisation had been

performed. Peptides were considered identified if the Mascot score was over 95% confidence limit.

### 2.3.8 Cloning and cDNA sequencing of complete prawn tropomyosin

#### 2.3.8.1 RNA extraction from Black Tiger prawns

100 mg of prawn meat was excised from a fresh catch of Black tiger prawns. It was then flash frozen in liquid nitrogen and crushed into fine pieces in a mortar and pestle. While still keeping the environment cold and not allowing it to thaw, the pulverised mass was transferred to a pre-cooled 1.7 mL tube. The RNA was then extracted using the RNeasy mini extraction kit (Qiagen, Hilden, Germany) by following the manufacturer's instructions. The extracted RNA was immediately used for the downstream processes or stored at -80°C.

#### 2.3.8.2 cDNA transcription and PCR amplification of tropomyosin

The Transcriptor High Fidelity cDNA Synthesis Kit was used for the transcription of cDNA from the extracted RNA, following the manufacturer's instructions. The oligo-dT primer was used for reverse transcribing the mRNA. The cDNA thus prepared was then used as a template for a standard PCR run. The primers were designed for Black tiger prawn tropomyosin (Uniprot number A1KYZ2). The primer sequences were as follows,

BTPTM\_forward primer\_ *BamH1*

GCGGATCC-GACGCCATCAAGAAGAAGATGC,

BTPTM\_reverse primer\_ *EcoR1*

GCGAATTC-TTAGTAG CCAGACAGTTCGCTG.

The PCR conditions were set as follows, 94 °C for 2 min, 30 cycles of 94 °C for 20 sec, 55 °C for 20 sec, 72 °C for 30 sec, 72 °C for 7min.

#### 2.3.8.3 Cloning of TM coding region into the sequencing vector, pCR2.1

The amplified PCR product, containing restriction sites for *BamH1* at 5' end and *EcoR1* at the 3' end, was digested using restriction enzymes; *BamH1* and *EcoR1*, respectively using the manufacturer's protocol (Promega, Madison,

Wisconsin, USA). The sequencing vector, pCR 2.1 was also digested using the same restriction enzymes. The subsequent digested products were separated on a 1% agarose gel in TAE buffer at 80V for 40 minutes. The bands relating to the digested products were excised from the gel and purified using the Wizard PCR prep, DNA purification system (Promega, Madison, Wisconsin, USA). The coding region for TM was ligated into the sequencing vector using T4 DNA ligase enzyme (Invitrogen, Carlsbad, CA, USA). For sequencing of the TM open reading frame, the purified recombinant plasmid, pCR2.1\_BTPTM was sent to Macrogen Inc, South Korea.

#### **2.3.8.4 Cloning of TM coding region into the expression vector, pRSET-A**

For recombinant protein expression, the PCR amplified product of the tropomyosin cDNA was cloned into an expression vector, pRSET-A using the protocol as detailed in Section 2.3.8.3.

#### **2.3.8.5 Transformation of BL21 competent cells by electroporation**

The recombinant expression vector was then transformed into electro competent *E.coli* BL21 cells for protein expression. A cuvette containing the expression vector and the *E.coli* cells was subjected to an electro pulse (200  $\Omega$ , 2.1-2.5 kv). SOC medium was added to this cuvette and immediately transferred into a 1.5 mL tube and shaken at 37 °C for 1hr. After incubation the cells were plated on LB agar containing 100 ug/mL ampicillin. After overnight incubation of these plates, several colonies were picked and checked for the tropomyosin insert using PCR technique. The positive colonies were inoculated into 10 mL of LB broth with 100 ug/mL and again grown overnight at 37°C. These clones were then kept at -80 °C as a glycerol stock.

### **2.3.9 Expression and purification of recombinant prawn tropomyosin**

#### **2.3.9.1 Culturing of BL21 Escherichia coli cells and induction of recombinant protein expression**

10 mL of a fresh overnight culture of the BL21 *E.coli* cells containing the recombinant expression vector were inoculated into 200 mL of LB broth

containing 100 ug/mL Ampicillin, and incubated at 37°C on shaking until the absorbance reached 0.3-0.5 at 600 nm. IPTG (Isopropyl  $\beta$ -D-1-thiogalactopyranoside) was then added to the culture to a final concentration of 1 mM to induce recombinant protein expression and further incubated for 3 hours.

### **2.3.9.2 Purification of rTM from the crude lysate using metal chelate chromatography**

The bacterial culture was centrifuged to pellet the cells and the supernatant was discarded. The cell pellet was resuspended in lysis buffer (25 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM imidazole, 2 mg/mL lysozyme). This mass was subjected to several freeze thaw cycles to lyse the cells and then treated with DNase (Qiagen, Hilden, Germany) to destroy the contaminant bacterial DNA. The crude bacterial lysate containing the recombinant protein was loaded onto a metal chelate affinity column charged with Ni<sup>+2</sup>. The column was washed with the washing buffer (25 mM Tris-HCl, pH 8.0, 300 mM NaCl) containing 25 mM imidazole to elute out contaminant proteins and the recombinant protein was eluted out using 250 mM Imidazole. The purified fractions of the recombinant protein were pooled and dialysed either against 100 mM ammonium bicarbonate buffer and freeze dried for further experimental use or dialysed against phosphate buffered saline, pH 7.2 for longer storage at -80°C.

### **2.3.10 Inhibition-ELISA**

An inhibition-ELISA was performed to analyse the mAb cross-reactivity to tropomyosin in the various shellfish extracts. A 96-well polystyrene high binding plate (Costar, USA) was coated with 0.1  $\mu$ g/well of recombinant tropomyosin for 4 hours at room temperature using carbonate buffer, pH 9.6, and subsequently blocked using 5% SMP in PBS-T. The mAb was mixed with increasing concentrations of inhibitors; raw and heated protein extracts of black tiger prawn, blue swimmer crab, rock lobster, blue mussel, scallop and squid and exposed to the coated wells for 1 hour at 37°C. A fish protein extract was used as a negative control. Antibody binding to the coated antigen was detected

using rabbit anti-mouse IgG antibody conjugated with HRP (Sigma, St. Louis, MO, USA) and visualised using 3,3',5,5'-tetramethylbenzidine (TMB) substrate for HRP (BD Biosciences, USA). The reaction was stopped using 2M sulphuric acid and the absorbance measured at 450 nm. Percent inhibition was calculated as  $100 - [(O.D.450 \text{ nm of antibody with inhibitor} / O.D.450 \text{ nm of antibody without inhibitor}) \times 100]$ .

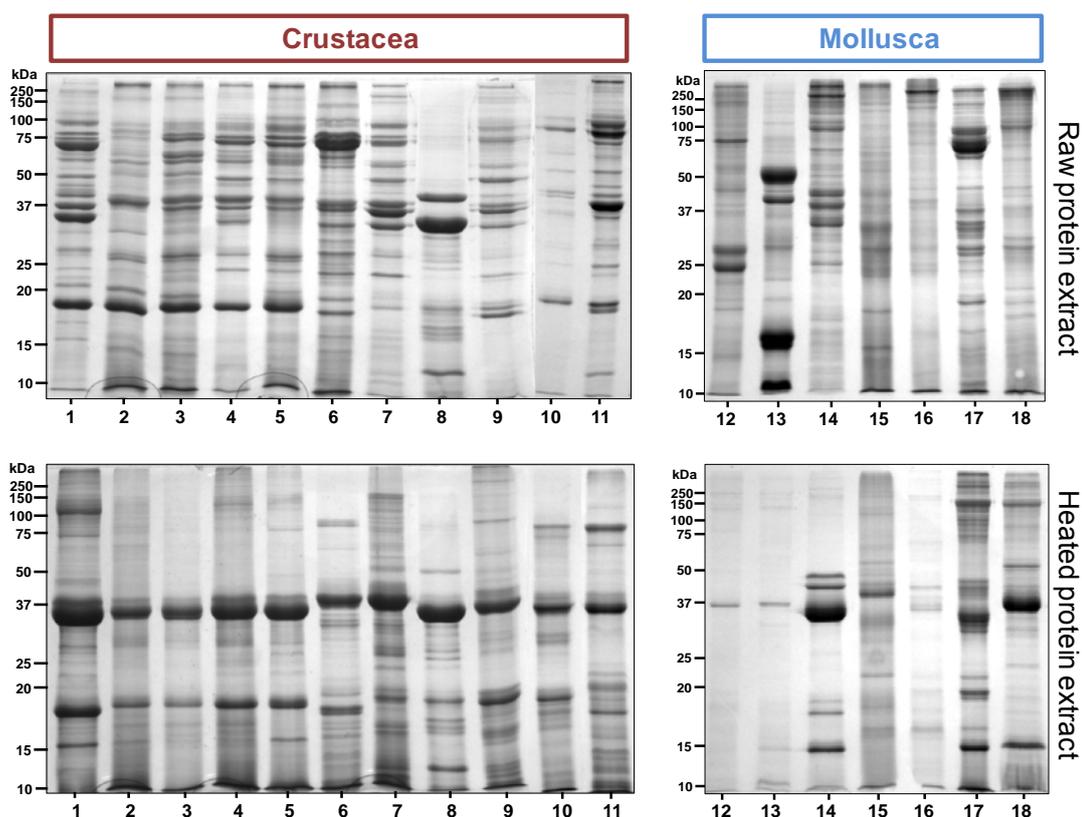
### **2.3.11 Amino acid sequence alignment of crustacean and mollusc tropomyosin**

An amino acid sequence comparison was performed using representative tropomyosin sequences from crustaceans; black tiger prawn (GenBank accession number, ADM34184.1), blue swimmer crab (GenBank accession number, JX874982), rock lobster (GenBank accession number, KC291442) and molluscs; green mussel (Genbank accession number, AAG08988.1), oyster (Genbank accession number, BAH10152.1) and octopus (Genbank accession number, BAE54433.1). Sequences were obtained from the National Centre for Biotechnology Information (NCBI). Multiple sequence alignment was performed using the ClustalW alignment in MEGA.

## 2.4 Results

### 2.4.1 Protein profile of shellfish extracts and the effect of heat treatment

Eleven crustacean and 7 mollusc samples listed in Table 1 were analysed for their protein repertoire in raw and heated extracts to evaluate the effect of heat treatment by 1D-SDS-PAGE (Figure 2.1). The raw extracts displayed a complex protein pattern with the majority of visible protein bands within the molecular weight range of 18 to 75 kDa. For crustaceans, the most prominent



**Figure 2.1:** SDS-PAGE analysis of raw and heated protein extracts of crustacean and mollusc species. Lanes 1 -11, crustacean extracts; 1, black tiger prawn, 2, king prawn, 3, vannamei prawn, 4, banana prawn, 5, green tiger prawn, 6, blue swimmer crab, 7, sand crab, 9, slipper lobster, 10, rock lobster, 11, yabby. Lanes 12-18, mollusc extracts; 12, green mussel, 13, blue mussel, 14, scallop, 15, oyster, 16, sea snail, 17 octopus, 18, squid. See Table 1 for complete list of scientific names for all species.

protein bands were in the range of 18 to 20 kDa and 35 to 40 kDa. Similarities could be observed among the SDS-PAGE profiles of the prawn group (lanes 1-5) and crab group (lanes 6-8), while there were variations in the profiles of the three lobster species (lanes 9-11). In the case of the mollusc raw extracts, the

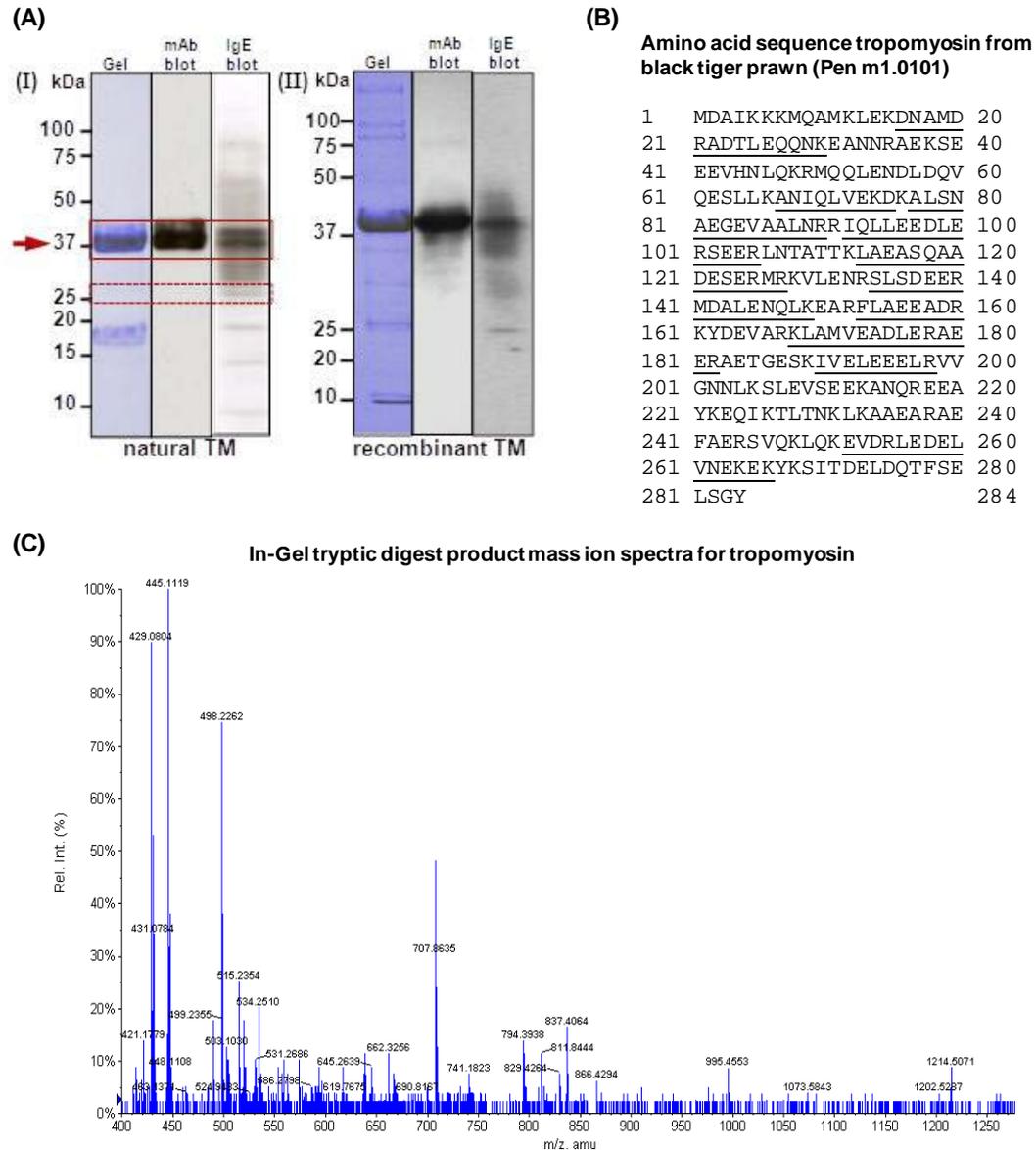
profiles varied considerably among each other with no identifiable protein banding pattern.

To analyse the effects of heat processing, the shellfish protein extraction parameters such as osmolarity, pH, extraction buffer volume, specimen weight, heating temperature and heating time were kept constant. This allowed direct comparison of the effect of heating on different shellfish species tropomyosin without any bias. The extracts of heat-treated shellfish displayed a more uniform protein-banding pattern. The heated crustacean extracts possessed major protein bands at about 18 kDa and 37 kDa, signifying the presence of heat stable proteins. Similarly, the heated mollusc extracts displayed a single major protein band at about 37 kDa, but with very different intensities.

#### **2.4.2 Purification and identification of tropomyosin and IgE reactivity**

In order to confirm specific monoclonal antibody (mAb) reactivity to tropomyosin, natural tropomyosin was purified from prawn protein extract using ion exchange chromatography. IgE antibody reactivity to natural tropomyosin was confirmed by immunoblotting against a pool of shellfish allergic patient sera (Figure 2.2 A). Mass spectrometric analysis confirmed the mAb and IgE antibody reactive protein to be tropomyosin by comparing generated peptides using the MASCOT database (Figure 2.2 C). In addition, a 26 kDa fragment of tropomyosin was detected which demonstrated IgE binding but was unable to bind to the mAb. The peptides identified in this fragment are shown in Appendix Table B1.1.

Subsequently this prawn tropomyosin was fully sequenced by cDNA analysis (Figure 2.2 B) and demonstrated greater than 89% amino acid identity with tropomyosin from the other investigated crustacean species. In contrast the amino acid identity to the six investigated mollusc tropomyosins was very low and ranged from 55% (green mussel) to 63% (octopus) (Appendix Figure B 2.1).



**Figure 2.2:** Purification and identification of tropomyosin and confirmation of mAb reactivity. (A) SDS-PAGE and immunoblotting profile of purified natural tropomyosin and recombinant prawn tropomyosin. Reactivity to mAb and patient sera IgE is shown respectively in (I) and (II). (B) Complete amino acid sequence of prawn tropomyosin. Peptides identified by mass spectrometry are underlined. (C) Precursor ion spectrum of mAb reactive tropomyosin digested and analysed using LC-MSMS; each peak represents a peptide that has been subsequently sequenced by MS-MS.

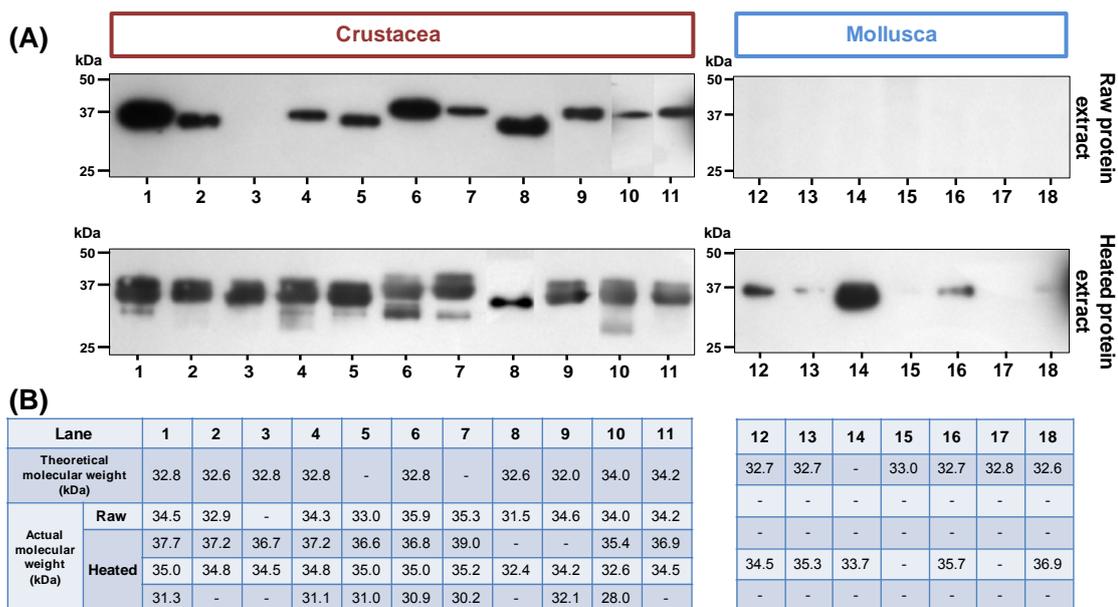
The generated recombinant tropomyosin (rTM) from black tiger prawn was further used as a standard for additional cross-reactivity studies (see Section 2.4.4).

### **2.4.3 mAb reactivity to tropomyosin in raw and heated shellfish extracts**

The raw and heated protein extracts of crustaceans and molluscs were evaluated for their mAb reactivity by immunoblotting (Figure 2.3). In the raw crustacean extracts, a single band was observed for each sample except for vannamei prawn. The molecular weight of these bands ranged from 31 to 36 kDa. Based on densitometric analysis of the bands, mAb reactivity was the strongest to black tiger prawn followed by blue swimmer crab and snow crab. Interestingly, none of the raw mollusc extracts reacted to the mAb.

The heated crustacean extracts demonstrated strong mAb binding in the range of 30 to 39 kDa. Compared to the raw extracts, the heated extracts showed mAb reactivity to multiple protein bands in each lane, differing in mass by an average of 2 kDa except for snow crab, which showed only one single band. Interestingly, in vannamei prawn the raw extract did not elicit any mAb reactivity, whereas strong binding was observed in the heated extract signifying an increase in antibody reactivity after heat processing.

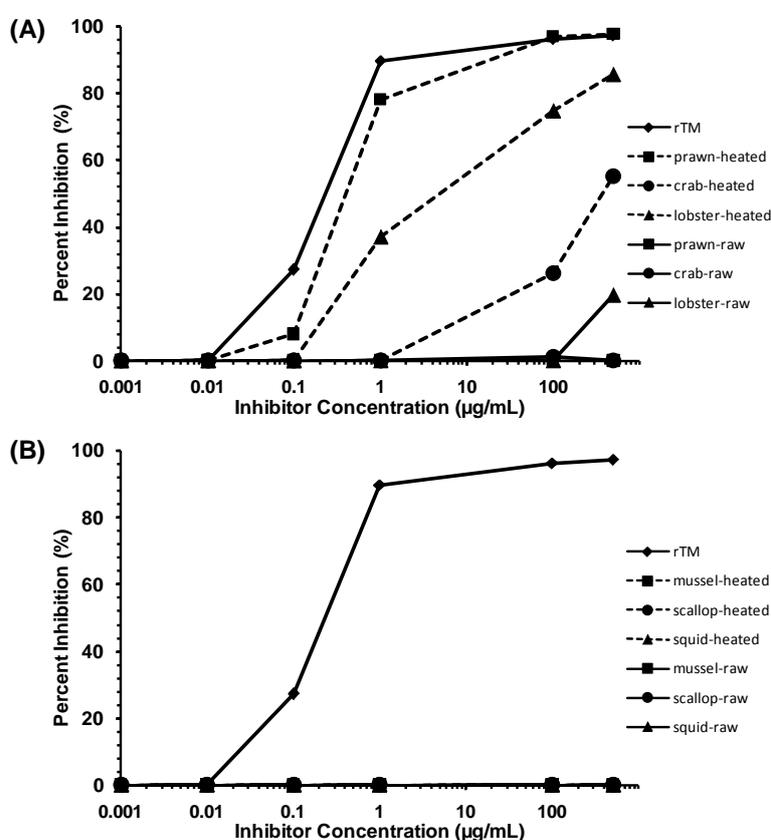
In contrast to raw mollusc extracts, heating increased mAb reactivity to some mollusc species. However, reactivity was only observed for green and blue mussel, scallop and sea snail, with very faint mAb reactivity for oyster and calamari and no reactivity to octopus protein extract.



**Figure 2.3:** Immunoblot analysis of raw and heated protein extracts of crustacean and mollusc species, using monoclonal anti-tropomyosin antibody. (A) Lanes 1–11, crustacean extracts; 1, black tiger prawn, 2, king prawn, 3, vannamei prawn, 4, banana prawn, 5, green tiger prawn, 6, blue swimmer crab, 7, sand crab, 8, snow crab, 9, slipper lobster, 10, rock lobster, 11, yabby. Lanes 12–18, mollusc extracts; 12, green mussel, 13, blue mussel, 14, scallop, 15, oyster, 16, sea snail, 17 octopus, 18, squid. See Table 1 for a complete list of scientific names. (B) Theoretical and actual molecular weights of tropomyosin variants detected in the raw and heated crustacean and mollusc extracts using the mAb.

#### 2.4.4 mAb cross-reactivity of recombinant tropomyosin against crustacean and mollusc extracts

To evaluate the cross-reactivity of the mAb to tropomyosin in raw and heated shellfish extracts quantitatively, an inhibition ELISA was performed using recombinant prawn tropomyosin as a standard (Figure 2.4). The decrease in reactivity of the mAb to immobilised tropomyosin on the ELISA plate was used as a measure of immunological cross-reactivity, using crustacean and mollusc protein extracts as inhibitors. Recombinant tropomyosin and heated extract from prawn was, as expected, able to completely abolish antibody reactivity at



**Figure 2.4:** Inhibition-ELISA for the quantitative analysis of cross-reactivity of the mAb between recombinant black tiger prawn tropomyosin and different crustacean (A) and mollusc (B), raw and heated extracts

less than 1 µg/ml. Lobster and crab heat treated extracts were able to inhibit mAb reactivity in a dose dependant manner, with the latter reaching only about 50% inhibition. In contrast, raw crustacean extracts, as well as raw and heated

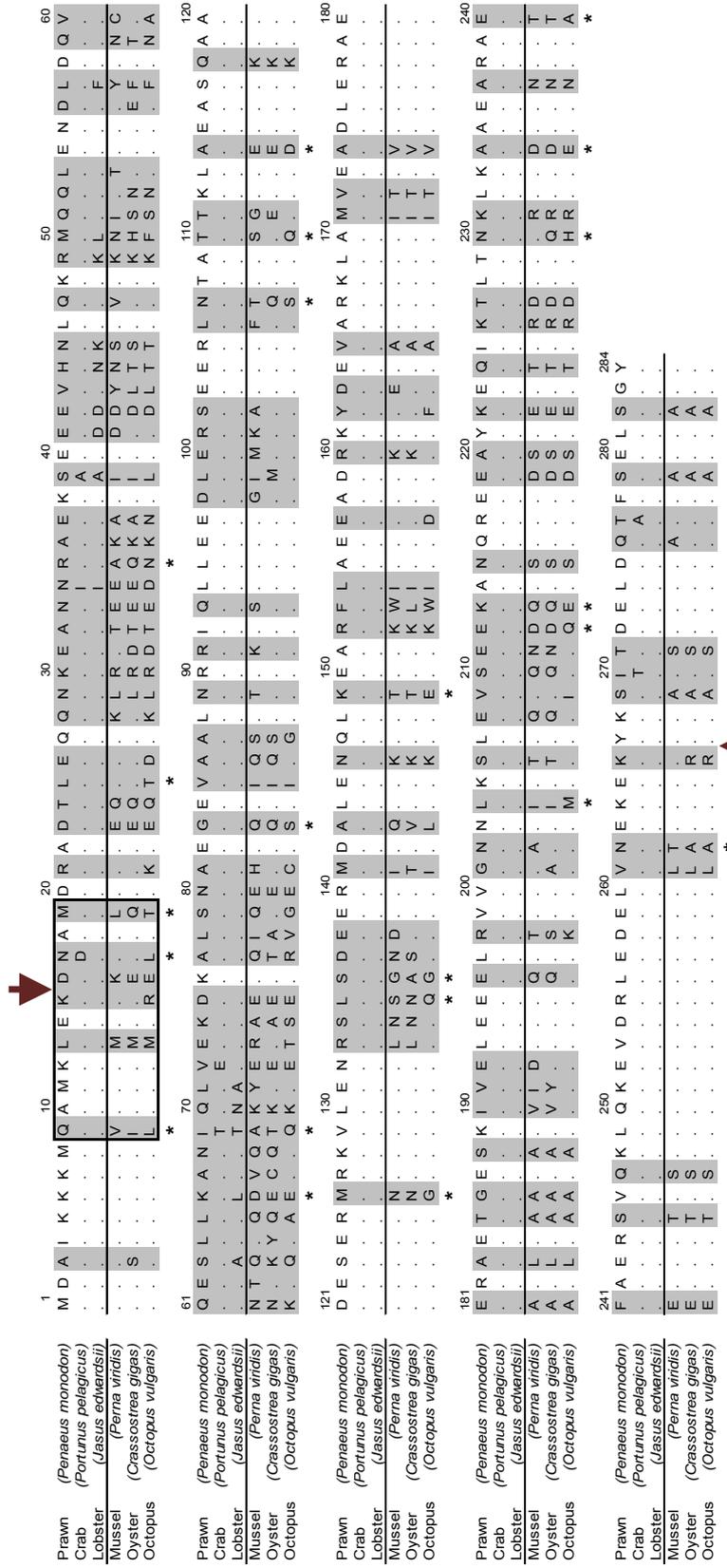
molluscs, were not able to achieve any significant inhibition even at the highest inhibitor concentration.

#### **2.4.5 Selective epitope recognition of mAb between crustacean and mollusc tropomyosins**

Based on the amino acid sequence alignment, 49% of tropomyosins' primary structure is conserved between the analysed crustaceans and molluscs (Figure 2.5). However, within the groups crustacean tropomyosins (prawn, crab and lobster) share over 89% sequence identity, while mollusc tropomyosins (mussel, oyster and octopus) share only 63% sequence identity within the individual species.

Based on the differential mAb reactivity to tropomyosin in the immunoblotting experiments and using amino acid sequence alignment from select crustacean and mollusc species, the prediction of the most likely binding site of the mAb on shellfish tropomyosin was attempted. Strong mAb binding to tropomyosin was demonstrated for prawn, crab, lobster, and mussel and no binding to oyster and octopus. This information was used to locate amino acid substitutions due to evolutionary changes in the primary structure along the entire protein, which could be responsible for changing the mAb reactivity. Twenty-two amino acid substitutions were identified on tropomyosin, fulfilling this criterion as shown by marked asterisk (Figure 2.5).

In addition, an approximately 26 kDa stable fragment of tropomyosin was identified using mass spectrometry in the heated prawn extract (Appendix Table B1.1), which was demonstrated to elicit IgE antibody binding, but lacked the ability to bind mAb. Based on the peptides matches, this fragment has been highlighted with its N- and C-terminal marked by red arrows (Figure 2.5). Therefore, it is predicted that the tropomyosin amino acid region 9-19 "QAMKLEKDNAM" is the most likely mAb binding epitope.



**Figure 2.5:** Amino acid sequence alignment of representative tropomyosin sequences from crustaceans (GenBank accession numbers); black tiger prawn (ADM34184), blue swimmer crab (JX874982), rock lobster (KC291442) and molluscs; green mussel (AAG08988), oyster (BAH10152) and octopus (BAE54433). Variable amino acid regions of the proteins' primary structure are shaded in grey. The amino acid substitutions between crustacean and mollusc tropomyosin are denoted with “ \* “. The red arrows indicate the N- and C-terminal ends of the 26 kDa fragment of tropomyosin which does not bind to the mAb. The predicted mAb binding epitope of tropomyosin is identified by a solid box.

## 2.5 Discussion

Tropomyosin is the major shellfish allergen. Due to its primary role in muscle function, it is present in much higher quantity than other identified shellfish allergens. Moreover, the highly conserved primary structure is responsible for its allergenic cross-reactivity not only between crustaceans and molluscs but also other invertebrates such as mites and insects.<sup>14, 15</sup> Therefore, tropomyosin is a commonly used biomarker for the detection of shellfish allergens.<sup>16-20</sup>

The ability of tropomyosin to withstand heat treatment and most known forms of food processing techniques can be attributed to its exceptionally stable alpha helical coiled-coil secondary structure.<sup>21</sup> Effect of heat treatment on the reactivity of patient IgE antibody to tropomyosin from crustacean species has been previously discussed.<sup>22-24</sup> Nevertheless not much information is available on the effect of heat processing for specific detection of tropomyosin from various shellfish groups, particularly molluscs. Monoclonal antibodies are preferred to conventional polyclonal antibodies as the former bind exclusively to a specific epitope, on the antigen, usually comprising of not more than ten amino acids. Using a specific monoclonal antibody, it was demonstrated that heating of shellfish increases the antibody reactivity to tropomyosin. Furthermore, it was shown that heating can also cause molecular differences between tropomyosins from the different shellfish groups investigated.

Heat processing resulted in increased antibody detection of tropomyosin for crustacean and mollusc extracts. Multiple variants of tropomyosin were observed for crustacean extracts. Interestingly, while no antibody reactivity was seen for raw mollusc extracts, characteristic binding was observed after heating. This increase in the mAb reactivity to tropomyosin may have been caused due to conformational changes in the secondary structure due to heating.<sup>25</sup> Yet another observation made was presence of higher molecular weight tropomyosin bands. These may be attributed to the phenomenon called as “Maillard reaction” which occurs due to chemical interaction of amino acid residues with sugar moieties at elevated temperatures. This phenomenon has been previously reported for shellfish and peanut allergens.<sup>26-28</sup> Tropomyosins being rich in lysine residues may readily react with reducing sugars at elevated temperatures resulting in the formation of Maillard products. Further studies

focusing on the observed tropomyosin variants in the current study are needed to confirm the presence and impact of Maillard products after heating of shellfish.

Several assays have been developed for the sensitive detection of tropomyosin in food matrices.<sup>18, 20</sup> However, there is a lack of analytical tools that can be employed for specific detection of tropomyosin from various shellfish species. This study demonstrates for the first time, the use of a commercially available monoclonal anti-tropomyosin antibody for the detection of tropomyosin in an extensive panel of crustacean and mollusc species. While all eleven crustacean tropomyosins demonstrated reactivity, not all mollusc tropomyosins were detected using this mAb. This lack of antibody recognition to mollusc tropomyosin was further confirmed using immunoblotting and inhibition ELISA, with both raw and heated mollusc extracts failing to inhibit mAb binding to prawn tropomyosin.

One of the main objectives of this study was to characterise the tropomyosin antibody binding region of a commercially available anti-tropomyosin mAb, which could possibly be used for the detection of the major shellfish allergens in food products as part of international food safety regulations. For this reason, black tiger prawn tropomyosin was selected for the detailed cloning and sequencing experiments to deduce its monoclonal antibody binding site and to subsequently extrapolate this information to other tropomyosins from crustaceans as well as molluscs. The recombinant form of tropomyosin from black tiger prawn was subsequently generated and its antibody binding was evaluated. The application of this recombinant protein as an allergen standard will enable future improved quantification approaches for the detection of the major shellfish allergen using specific antibodies.

As the next step, the prediction of the mAb binding site on tropomyosin was attempted. Three representative crustacean and mollusc species were selected, since the tropomyosin sequence identity was high within these two sub-groups. Published amino acid sequences from GenBank were compared, in order to understand the underlying molecular characteristics of tropomyosin across the different species. There were 22 specific amino acid substitutions

along the primary structure of tropomyosin. Three major amino acid substitutions were revealed near the N-terminal of the protein by amino acid sequence comparison. Moreover an identified tropomyosin fragment (residue 16-266) was able to elicit IgE binding but exhibited no mAb reactivity. This indicated the possibility of the presence of a specific mAb epitope at the N- or C-terminal of the protein. Based on the antibody binding data and using amino acid sequence analysis, the most likely mAb binding epitope was identified to be located between amino acid residues 9 to 19.

In summary, it was shown that heating has a profound effect on the detection of the major shellfish allergen tropomyosin, which could have considerable implications for the detection and quantification of tropomyosin in processed food. Further studies are needed to characterise the multiple tropomyosin variants formed during heating or various “cooking” processes. In this study, the analytical application of a specific anti-tropomyosin antibody for the differentiation of tropomyosin from crustaceans and molluscs at a molecular level was successfully demonstrated. While this mAb was able to detect tropomyosin from all crustacean species tested, it detected few mollusc tropomyosins. A specific antibody target was identified in the N-terminal region of shellfish tropomyosin to enable the differentiation between crustacean and mollusc allergens.

More specific assays may be developed by applying this immunological approach in detecting shellfish tropomyosin for better food labelling for consumer protection. The next chapter investigates the development and application of a chemical-based mass spectrometric approach instead of antibody-based immunoassays for the detection and quantification of shellfish allergens in processed food. The basis for the differential recognition of tropomyosin from different shellfish species is most likely due to the different molecular characteristics and amino acid sequences. Further studies on the amino acid sequences of different shellfish tropomyosins and possible post-translational modifications will help us to understand the different immunological reactivities of this major food allergen.

## 2.6 References

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## 2.7 Chapter 2 summary:

- The aim of this study was to analyse an extensive panel of crustacean and mollusc species for the major allergen tropomyosin using a commercial monoclonal antibody
- Using monoclonal antibodies, tropomyosin was detected in all eleven crustacean species, with partial binding to tropomyosin in molluscs; mussels, scallops and snails and no binding in oyster, octopus and squid
- Heating of shellfish has a profound effect on the detection of tropomyosin. This was evident by the enhanced recognition of multiple tropomyosin variants in the analysed shellfish species
- The antibody binding site on tropomyosin was predicted in the amino acid region 9 to 19

## CHAPTER 3

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### ANALYSIS OF THE ALLERGENIC PROTEINS IN BLACK TIGER PRAWN (*PENAEUS MONODON*) AND CHARACTERISATION OF THE MAJOR ALLERGEN TROPOMYOSIN USING MASS SPECTROMETRY

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### 3.1 Introduction

Black tiger prawn (*Penaeus monodon*) is one of the most important commercial products of the seafood industry in south-east Asia and is widely distributed in the Indo-West-Pacific region.<sup>1</sup> The seafood industry has experienced tremendous growth in recent years with over 42 million workers worldwide engaged in various activities of seafood production (i.e. processing, harvesting, etc.) and these workers are being continuously exposed to seafood allergens.<sup>2</sup> Tropomyosin<sup>3</sup> has been identified as an immunologically reactive protein from Black tiger prawn (BTP) in previous studies using molecular and genetics approaches. Immunochemical techniques have been used in previous studies to detect allergenic proteins in food products or to quantitate air-borne allergens in air samples including enzyme-linked immunosorbent assay (ELISA)<sup>5, 6</sup> radioallergosorbent tests (RAST),<sup>7</sup> and immunoblotting.<sup>6, 8</sup> However, issues such as matrix interference and inter-assay variations affect the sensitivity and reproducibility of such assays. Chemical-based approaches such as mass spectrometric techniques on the other hand are highly sensitive and accurate and have been used successfully for the identification and characterisation of various proteins.<sup>9, 10</sup> Recent studies have focused on the characterisation of specific or unique regions of allergenic proteins called signature peptides. These peptides are being increasingly used in the detection and quantification of allergens from different food sources using mass spectrometric approaches. New advances in mass spectrometric analysis of proteins have enabled the complete sequencing of proteins using enzymatic digestion methods and identification of the generated peptides mass, called *de novo* sequencing.

In Chapter 2, tropomyosin was identified from 17 different shellfish species using immunological methods with specific monoclonal antibodies. Black tiger prawn tropomyosin was expressed and purified as a recombinant protein as a tool for further immunological analysis. cDNA sequencing however is possible only for proteins with known amino acid sequences in various databases. Moreover, the use of recombinant allergens synthesized in prokaryotic expression systems does not take into account, the post translational modifications. These modifications may play a role in the allergen IgE binding

capacity and antigenicity and it is important to identify such factors to facilitate the use of allergen components for improved diagnosis.

Identification and quantification of major prawn allergens require a highly sensitive, specific, and reproducible analytical technique. Isotopic dilution mass spectrometry has played a crucial role in protein quantification in the last two decades. This technique was the method of choice for further studies in the characterisation and quantification of crustacean allergens.

The current chapter details the identification and characterisation of Black tiger prawn allergens using mass spectrometric techniques. IgE binding allergenic proteins were detected in Black tiger prawn using sera from shellfish allergic patients. A very specific and selective method was developed to both characterise and quantify these allergens using mass spectrometry whereby specific allergenic proteins were targeted rather than a group of reactive but uncharacterised proteins. This enabled identification of the actual allergen and subsequent quantification using isotopic dilution mass spectrometric techniques. In this chapter, a novel signature peptide was characterised for Black tiger prawn tropomyosin. Moreover, tropomyosin was completely sequenced and analysed by *de novo* sequencing using a bottom-up approach.

### 3.2 Aims

The aims of this study were as follows,

- 1) To develop and standardise mass spectrometric methods for the identification and characterisation of IgE antibody binding proteins.
- 2) To perform *de novo* sequencing of the major prawn allergen, tropomyosin using a bottom-up Tandem MS approach.
- 3) To design a signature peptide for Black tiger prawn tropomyosin for allergen detection and quantification.

### 3.3 Materials and Methods

#### 3.3.1 Chemicals and reagents

All chemicals were used without further purification. Acetonitrile, hydrochloric acid, and methanol were supplied from ACP (Montreal, Canada). RapiGest SF surfactant was purchased from Waters Corporation (Milford, MA, USA) and trypsin, Asp-N and endoproteinase (Glu-C V8) sequencing grade enzymes from Sigma-Aldrich (St. Louis, MO, USA). Ammonium bicarbonate, O-methylisourea hemisulfate, ammonium hydroxide, horseradish peroxidase (HRP), and a-cyano-4-hydroxycinnamic acid (HCCA) matrix were purchased from Sigma-Aldrich. The Bradford assay kit was purchased from BioRad (Hercules, CA, USA). The dialysis bags were from Fischer Scientific (Roncho Dominguez, CA, USA). For desalting purposes, ZipTip C18 filters were purchased from Millipore Corporation (Bedford, MA, USA). For TM purification steps, phosphate-buffered saline (PBS) and Tris buffered saline (TBS) tablets were purchased from Amresco, USA. Acetic acid, sodium acetate, sodium chloride, and Tween-20, used for the washing steps, were purchased from Sigma-Aldrich. The Amicon spin filters used for fraction concentration were from Millipore Corporation. The tracker dye (Coomassie stain R250) and PVDF membrane for the immunoblotting were from BioRad (Hercules, CA, USA). Skimmed milk for the immunoblotting procedure was purchased from a local supermarket.

#### 3.3.2 Protein extracts

Fresh Black tiger prawns were purchased from a local market (Victoria, Australia) and transported to the laboratory on ice. Protein extraction was performed as detailed in Section 2.3.2. Briefly, the prawn muscles were shredded into pieces and homogenised in PBS, pH 7.2, using an Ultra turrax homogenizer (IKA, Germany) for generation of the raw protein extract. The slurry was then centrifuged at 8000 rpm for 20 min and the supernatant was filter sterilised. This PBS protein extract was stored in aliquots at 80°C until further experiments. Cooked prawn extract was prepared by heating whole

prawns at 100°C in PBS, pH 7.2, for 20 min, and then a similar procedure was followed as described for the raw prawn protein extract.

### 3.3.3 Tropomyosin purification

Tropomyosin was purified from the Black tiger prawn as detailed in Section 2.3.6. The tropomyosin from BTP was purified from the crude (raw and cooked) extract using a strong anion-exchange chromatographic column on a Biologic LP purification system (BioRad, USA). Before loading the proteins onto the column, the crude extract was exchanged into the chromatographic starting buffer (30 mM acetate buffer, pH 5.5) using Amikon spin filters of 3 kDa molecular weight cut off (MWCO). After equilibrating the column with the starting buffer, approximately 10mg of crude proteins were loaded onto the column. The column was then washed with 5 column volumes of the starting buffer. It was then further washed with 250mM NaCl in 30mM acetate buffer, pH 5.5, to elute unwanted proteins. The tropomyosin (TM) eluted at approximately 47 min. The collected fraction was then concentrated using an Amikon spin filter with a 3 kDa MWCO.

### 3.3.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The BTP crude extracts and the purified TM were profiled using 12% SDS-PAGE. A protein solution (10 mg) was added to each of the wells, and electrophoresis was run at a voltage of 170V for 1 h, or until the tracker dye was seen at the base of the gel. One gel was stained with Coomassie brilliant blue using a standard protocol. For the second gel, the separated proteins were transferred to a nitrocellulose membrane at 100V for 1 h. After the transfer was completed, the membrane was placed in a blocking solution (5% skimmed milk in TBS) for immunoblotting.

### 3.3.5 Immunoblotting

After separating the proteins using SDS-PAGE, samples were immobilised on a PVDF membrane using a semi-dry immunoblot apparatus (BioRad, USA). The membranes were then blocked with 5% skimmed milk solution for 1 hour at room temperature. To demonstrate the allergenicity of the prawn proteins, the different prawn extracts were analysed for IgE antibody binding from allergic patients. The human sera were collected from patients with strong allergic reactivity to shellfish. Ethics approval for this study was granted by James Cook University's Ethics committee (Project number H4313) in collaboration with The Alfred Hospital (Project number 192/07) and Monash University's Ethics Committees (MUHREC CF08/0225). For immunoblotting, protein extracts were separated by electrophoresis (see SDS gels above), proteins transferred and incubated with human serum (diluted 1:20 in 1% skimmed milk) overnight at 4°C. Subsequently, blots were washed three times with PBS-T and the membrane incubated for 1 hour with rabbit anti human IgE antibody (DAKO, USA) (diluted 1:1000) in PBS-T containing 1% skimmed milk. After washing the membrane with PBS-T three times, it was incubated for 30 min with HRP tagged goat anti-rabbit polyclonal antibody (DAKO,USA) (diluted 1:1000) in PBS-T containing 1% skimmed milk. Finally, the membrane was washed with PBS three times, incubated with the chemiluminescent substrate, and the immunoblot membranes analysed for IgE reactivity using the ECL technique.<sup>6</sup>

### 3.3.6 Enzymatic digestion and guanidation

The examined protein bands were excised from the SDS-PAGE plate. To increase the sensitivity of the lysine-containing peptides in the MALDI experiments, the in-gel guanidation procedure was performed on all protein samples using the protocol developed by Sergeant *et al.*<sup>11</sup> The gel pieces were destained by washing three times with 200mM of  $(\text{NH}_4)_2\text{CO}_3$  in a solution of 50% acetonitrile in  $\text{dH}_2\text{O}$ , at 30°C for 30 min. The destained pieces were dried under a stream of  $\text{N}_2$ , then covered by a solution of 50mM  $(\text{NH}_4)_2\text{CO}_3$ , pH7.8, containing 5 ng/mL trypsin, Asp-N, and endoproteinase Glu-C V8 in ice for 30 min for rehydration. After rehydration, any excess solution was removed. The

gel was then covered by a solution of 50mM of  $(\text{NH}_4)_2\text{CO}_3$  and incubated at 37°C overnight to enhance protein digestion. The water-soluble peptides were extracted twice with the incubation solution and other remaining peptides extracted twice with 0.15% trifluoroacetic acid (TFA) in 50% ACN after vortex mixing for 2 min. The samples were freeze-dried, and reconstituted prior to analysis with 10mL of 0.1% TFA and desalted with C18 ZipTips. An in-solution digestion for the pure extract of the BTP TM was performed using RapiGest SF surfactant<sup>12</sup>, which enhances the digestion efficiency. Subsequently, the solution was incubated with proper buffers as in the in-gel digestion protocol overnight with a concentration 20 ng/mL of enzyme. The digestion was quenched and the surfactant was precipitated by 1% formic acid at room temperature.

### 3.3.7 MALDI plate preparation

The protein samples were prepared for MALDI analysis using a protocol developed by Abdel Rahman *et al.*<sup>13</sup> Two layer sample/matrix preparation was employed for plate spotting. The first layer solution consisted of 20mg/mL HCCA in (1:9) methanol/acetone. The second layer of solution consisted of saturated HCCA in 40% ACN. A 0.5 mL sample of the first layer of matrix solution was applied to a MALDI target. A 1 mL sample of the second layer matrix solution was mixed with 1 mL of sample. Finally, 1mL of the sample/matrix mixture was deposited onto the first layer and allowed to dry, followed by an on-target wash step.<sup>13</sup>

### 3.3.8 MALDI- and ESI-QqToF MS

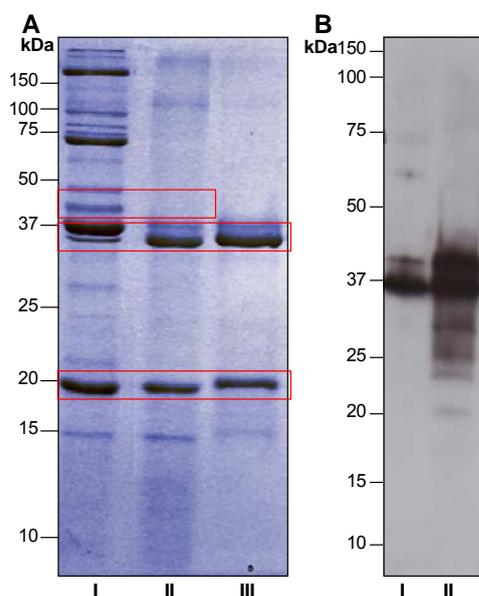
MALDI-MS and low-energy collision-induced dissociation (CID) analyses were carried out on a QSTAR XL hybrid quadrupole-quadrupole/time-of-flight tandem mass spectrometer (QqToF-MS/MS; Applied Biosystems/MDS Sciex, Foster City, USA) equipped with an o-MALDI ion source (Applied Biosystems, Foster City, CA, USA). Peptide separation was conducted using a DIONEX UltiMate3000 Nano LC system (Germering, Germany). A 250 fmol sample of

protein digest dissolved in 0.1% TFA was loaded onto a pre column (300 mm i.d. 5mm, C18 PepMap 100, 5 mm; LC Packing, Sunnyvale, CA, USA) for desalting and concentrating. Peptides were then eluted from the pre column and separated on a nano-flow analytical column (75mm i.d. 15 cm, C18 PepMap 100, 3mm, 100 Å; LC Packing) at 180 nL/min using the following gradient. The aqueous mobile phases consisted of (A) 0.1% formic acid/ 0.01% TFA/2% ACN and (B) 0.08% formic acid/0.008% TFA/98% ACN. A gradient of 0% B for 10 min, 0–60% B in 55 min, 60–90% in 3 min, 90% B for 5 min was applied. Including a regeneration step one run was 106 min long. The ESI mass spectra of the LC-eluting peptides were measured with the same hybrid QqToF-MS/MS system equipped with a nano-ESI source (Protana manipulator). The nano electrospray was generated from a Pico Tip needle (10mm i.d.; New Objectives, Woburn, USA) at a voltage of 2400 V. Individual target proteins were further analysed by CID-MS/MS; the resulting peptides were *de novo* sequenced and the results confirmed by using the National Centre for Biotechnology Information non-redundant database (NCBI nr) with the Matrix Science (Mascot) search engine (precursor and product ion mass tolerance set at 0.2 Da). Methionine oxidation was allowed as a variable modification and guanidinylation (K) as a fixed modification since the guanidination derivatisation has been performed in MALDI experiments. Peptides were considered identified if the Mascot score was over the 95% confidence limit.

### 3.4 Results

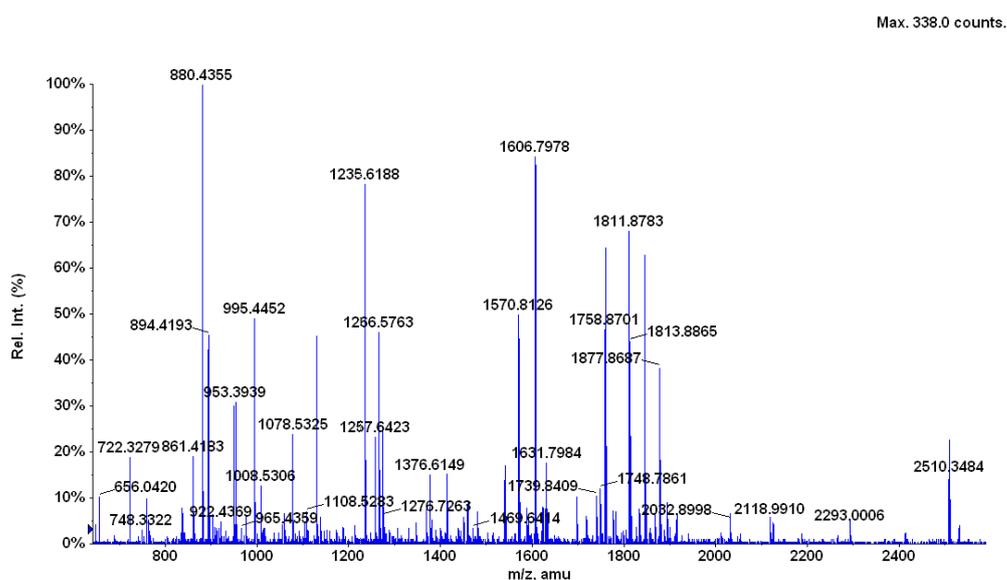
#### 3.4.1 Identification of IgE binding prawn proteins using immunoblotting

The crude protein extracts were isolated from fresh prawn tissues then profiled by SDS-PAGE (Figure 3.1 A) and transferred to nitrocellulose membranes for Western blotting. Subsequently, the blots were incubated with sensitised patient sera (Figure 3.1 B). This permitted the examination of the immune-reactivity of the extracted proteins. The strong IgE reactive band with a MW 33 kDa was further characterised using peptide mass finger printing (PMF). The 33 kDa band was excised and exposed to trypsin digestion. The generated peptides were subjected to MALDI-MS and LC/ESI-MS analyses. The precursor ions spectra (Figure 3.2) were uploaded to the Mascot search engine NCBI nr databases, where the sequences of the proteins were *in silico* generated. The Mascot search engine scored 112 identifying this band as a *Penaeus monodon* tropomyosin.



**Figure 3.1:** Immunochemical profile of the various protein extracts of the Black tiger prawn, I- Raw muscle extract, II- Heat treated muscle extract, III- Purified fraction of TM. A- SDS-PAGE Coomassie profile of the prawn extracts, B- IgE antibody immunoblot against Black tiger prawn, raw extract and heat treated extract using a pool of patient sera. The red box indicates the position of (top to bottom) Arginine kinase, Tropomyosin and Myosin Light Chain respectively.

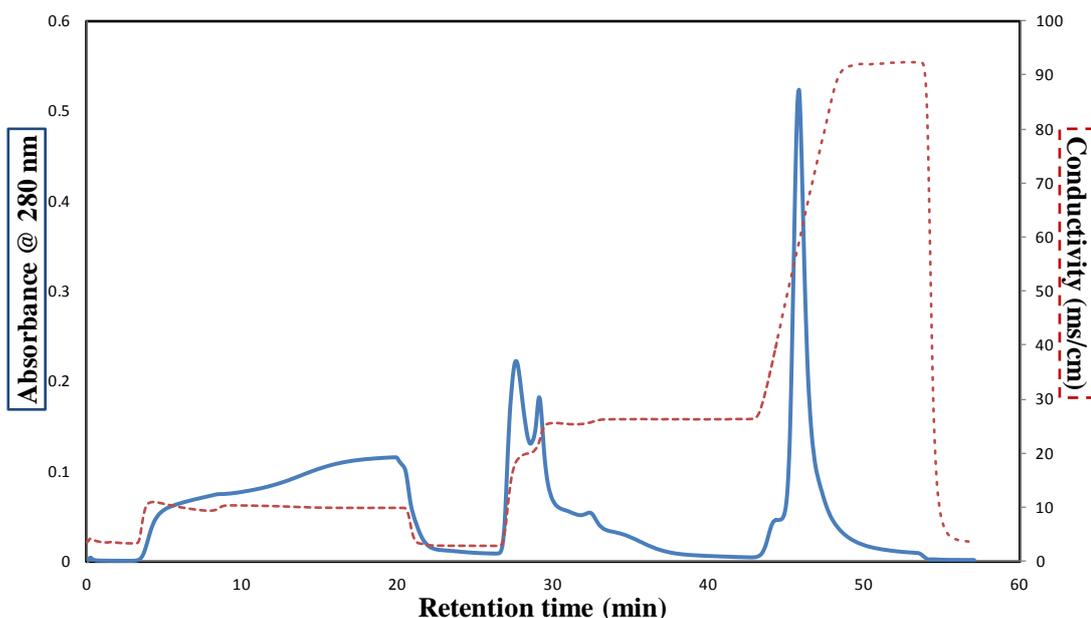
This represents a top probability based on Mowse scores matching with tropomyosin (complementary deoxyribonucleic acid (cDNA) based library; NCBI nr). The above scores were matched with the Mascot algorithms' criterion; individual ions scores >82 indicate identity or extensive homology ( $p < 0.05$ ). Subsequently, tropomyosin was isolated from Black tiger prawn and purified using an effective ion-exchange chromatography protocol (see Experimental section and Figure 3.3). The IgE antibody binding capacity of tropomyosin was further analysed using a pool of allergic patient sera, as shown in Figure 3.1 B.



**Figure 3.2:** Precursor ion spectrum generated after tryptic digestion of BTP TM. The spectrum scored 112 in the Mascots algorithm as TM for Black tiger prawn.

### 3.4.2 *De novo* sequencing of Black tiger prawn tropomyosin

Further characterisation was necessary to understand the structural biochemistry of this major allergen. The purified TM extract was *de novo* sequenced using bottom up approach peptide fragment fingerprinting. Various sets of enzymatic peptides were produced by trypsin, Glu-C V8, or Asp-N digestions and individually introduced into both the MALDI and ESI ion sources of a tandem mass spectrometer. In the precursor ion spectra, the most

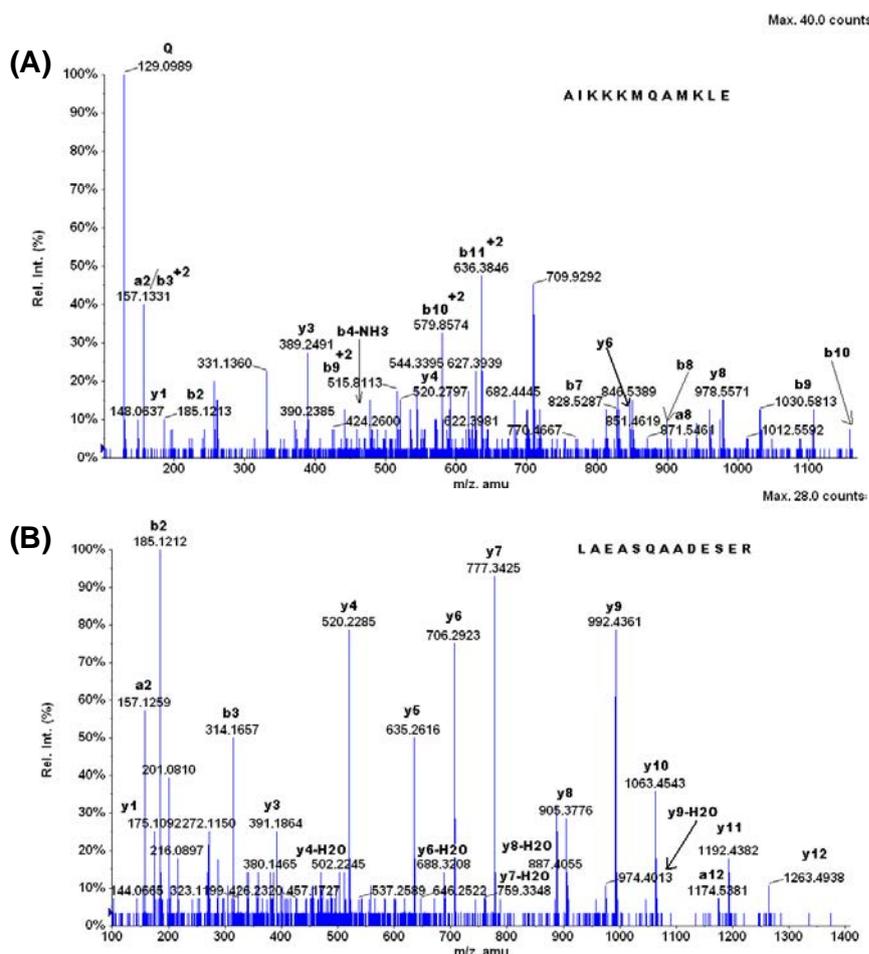


**Figure 3.3:** Ion-exchange chromatogram representing the purification of tropomyosin from BTP. The increase in the absorption (in A.U.) at 280nm (blue or solid line) at 47 min represents the elution of the tropomyosin fraction with increasing salt concentration (red or dashed line; conductivity in mS/cm).

abundant peptide ions (i.e.,  $m/z$  721.2927  $[M+H]^+$ ,  $m/z$  879.3927  $[M+H]^+$ ,  $m/z$  949.3927  $[M+H]^+$ ,  $m/z$  1060.4927  $[M+H]^+$ ,  $m/z$  1107.4927  $[M+H]^+$ ,  $m/z$  1128.4927  $[M+H]^+$ ,  $m/z$  1136.4927  $[M+H]^+$ ,  $m/z$  1145.5927  $[M+H]^+$ ,  $m/z$  1156.5927  $[M+H]^+$ ,  $m/z$  1211.4927  $[M+H]^+$ ) were selected sequentially in the gas phase and exposed to low-energy CID.

The peptide fragment ions were further separated by the TOF analyser with respect to their mass-to-charge ratio. In LC/ESI-QqToF analyses, the peptides were initially separated with respect to their polarity on the nano-HPLC column (C18 PepMap), using LC separation and a suitable solvent to minimize ion suppression and increase the abundance of ionised peptides.<sup>14</sup> Therefore, the multiply charged precursor ions (i.e.  $m/z$  457.7712  $[M+2H]^{+2}$ ,  $m/z$  539.7791  $[M+2H]^{+2}$ ,  $m/z$  565.3142  $[M+2H]^{+2}$ ,  $m/z$  606.7909  $[M+2H]^{+2}$ ,  $m/z$  629.3417  $[M+2H]^{+2}$ ,  $m/z$  688.8189  $[M+2H]^{+2}$ ,  $m/z$  695.3667  $[M+2H]^{+2}$ ,  $m/z$  514.2560  $[M+3H]^{+3}$ ,  $m/z$  1016.0235  $[M+2H]^{+2}$ ) were selected and isolated for low-energy CID experiments. The fragments of these peptides were separated by the ToF mass analyser.

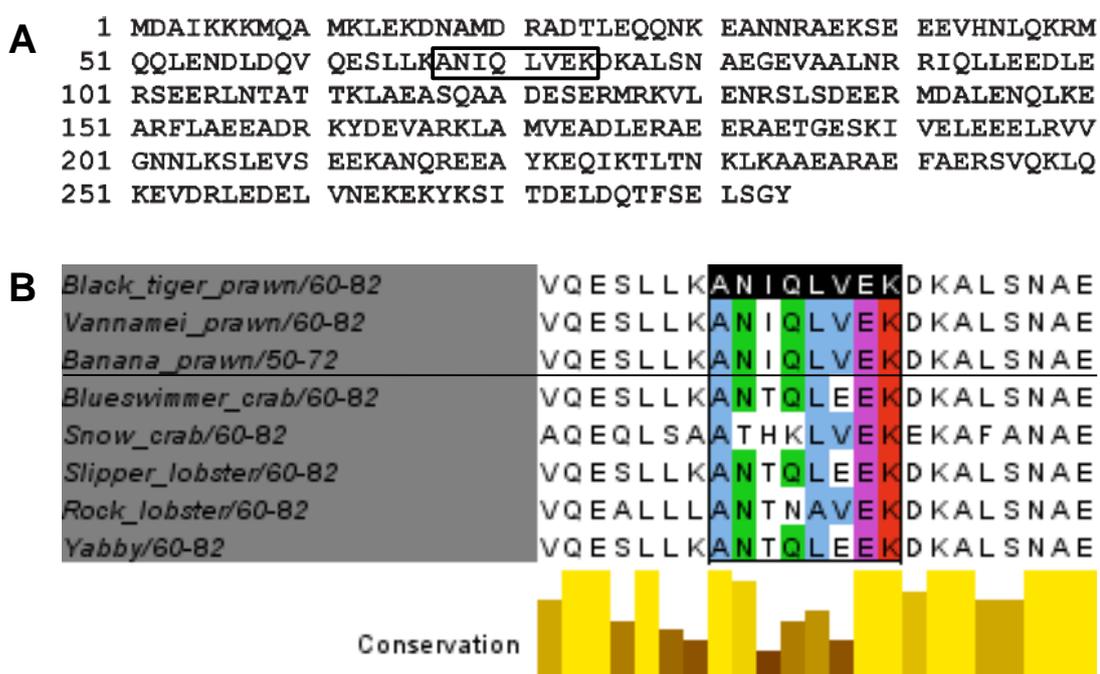
The product spectra generated by both ionization mass spectrometry techniques were uploaded to the Mascot MS/MS search engine against the NCBI nr database. The Mowse scores for these runs were on average 1461, 481, and 187 as Black tiger prawn tropomyosin identification for the in-solution generated peptides by trypsin, Glu-C V8, and Asp-N digestion, respectively. Appendix Table B1.2 reports the only peptides that match the Mascot criteria for the individual ions along with expected and calculated molecular weight. Elucidated representative MS/MS spectra for two peptides are shown in Figure 3.4, where the most dominant peptide fragment ions (y and b ions) are highlighted.



**Figure 3.4:** A representative product spectra of two selected peptides (A) generated from Glu-C V8 digestion and the ESI ion source for  $m/z$  709.9323  $[M+2H]^{+2}$  with sequence AIKKKMQAMKLE and (B) generated from tryptic digestion and the MALDI ion source for  $m/z$  1375.6215  $[M+H]^+$  with sequence LAEASQAADER.

Multiple types of enzymatic digestions, ion sources, and derivatisation protocols were applied to maximize the amino acid coverage of tropomyosin.<sup>15</sup> As stated, three different sets of enzymatic peptides were generated from BTP TM by trypsin, Asp-N, and Glu-C (V8) proteases. These different peptides with different termini enlarge the amino acid sequence coverage probability of the entire protein. A list of those peptides were organized in order as listed in Appendix Table B1.2. The full amino acid *de novo* sequence for BTP TM is shown in Figure 3.5. In MS studies, peptides containing the N-terminal methionine are difficult to ionise by ESI ion sources due to PTM acetylation on the amino acid. The evaluation of this PTM in prawn tropomyosin by the NetAcet 1.0 server scored a high value of 0.471.<sup>16</sup> Acetylation modification is

quite common for eukaryotic TM, as reported for bovine, chicken (P04268) and human (P09493) cases in the UniProtKB/Swiss-Prot databases.<sup>13</sup> In another case with MS sensitivity, the MALDI ion source is highly selective for the arginine-containing peptides. Therefore, a guanidation reaction was performed to increase the sensitivity of the lysine-containing peptides in MALDI work. The guanidation reaction chemically modifies the lysine side chain to homoarginine, which has a proton affinity equivalent to the arginine residues resulting in better sensitivity using the MALDI source.



**Figure 3.5:** (A) Black tiger prawn tropomyosin amino acid *de novo* sequence using the bottom up Tandem MS approach. (B) The prawn tropomyosin signature peptide “ANIQLVEK” (shaded black) compared to tropomyosin sequences from other crustacean species from Genbank; Vannamei prawn (*Litopenaeus vannamei*), Banana prawn (*Fenneropenaeus merguensis*), Blueswimmer crab (*Portunus pelagicus*), Snow crab (*Chionocetes opilio*), Slipper lobster (*Thenus orientalis*), Rock lobster (*Jasus edwardsii*) and Yabby (*Cherax destructor*). The degree of conservation for each amino acid position is represented as shaded bars. Multiple sequence alignment was performed using Jalview

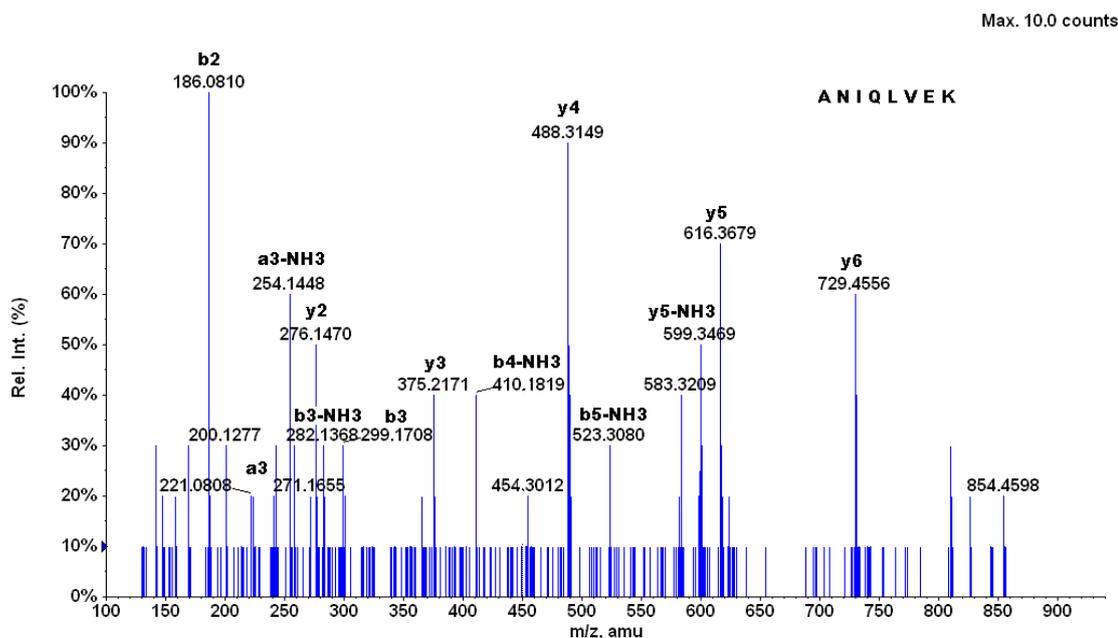
### 3.4.3 Identification of other Black tiger prawn allergens

Additional BTP proteins have been characterised in this study using mass spectrometry. A number of their enzymatic peptides were sequenced using the same MS strategy as described for tropomyosin. The 20-kDa band, as shown in Figure 3.1, and which demonstrated IgE binding, was excised and characterised. Two relevant and abundant peptides of this band were *de novo* sequenced using PFF. The amino acid sequences for these peptides were EGFQLMDR and GTFDEIGR and searched against the NCBI nr databank using the Mascot search engine. These peptides matched with myosin light chain, a recently reported allergen in white leg shrimp (*Litopenaeus vannamei*; Lit v3).<sup>17</sup> The BTP arginine kinase was also characterised as the 40-kDa protein which also identified as an IgE reactive protein. The band was excised, tryptic digested, and analysed by MS. The most abundant peptides were *de novo* sequenced (i.e. AVFDQLKEK, VSSTLSSLEGELK, GTYYPLTGMSK, LIDDHFLFK, IISMQMGGDLGQVFRR, LTSAVNEIEKR, IPFSHHDR, GTRGEHTEAEGGIYDISNK). Additional muscle proteins such as troponin C, myosin heavy chain and calmoduli were also profiled by mass spectrometry in the same manner as the arginine kinase and myosin light chain.

### 3.4.4 Identification of Black tiger prawn signature peptide

Another major objective of this study was to identify a suitable signature peptide as a surrogate for the BTP TM protein in quantitative measurements of TM in the seafood workplace. A very important factor for selecting a signature peptide is the absence of PTM groups (i.e. phosphorylation and glycosylation).<sup>18</sup> Therefore, the precursor ions and intensity data generated from PMF experiments were uploaded on the ExPASy FindMod tool to check if there was any potential peptide having any PTM motif(s). The report indicated the absence of any type of modifications. Further confirmation was obtained by manual searching for the calculated molecular ions of PTM motifs in the precursor spectra. Additional assessment was performed using the NetPhos 2.0 server.<sup>19</sup> Next, peptides that scored >0.50 in abundance rating were excluded as the signature peptide nomination for more suitable candidate

sensitivity in quantification. All resultant peptides from the tryptic digestion without any missing cleavage (Appendix Table B1.2) were examined for signature peptide criteria. The uniqueness of peptides was recommended by the Mascot search engine along with PTM evaluation from the NetPhos 2.0 server.<sup>19</sup> The selected peptides were then subjected to the protein BLAST test. The NCBI BLAST test, which is used to find regions of local similarity between sequences of the NCBI database and calculates the statistical significance of matches, reported that the peptide located at 67-74 (ANIQLVEK) would be a suitable signature peptide for Black tiger prawn TM (with 100% identity, score = 28.2 bits (590), and expected = 94). The product ion spectrum of this peptide,  $m/z$  457.7712  $[M+2H]^{+2}$ , was collected using an ESI source (Figure 3.6), where the most abundant  $y$  and  $b$  ions were assigned.



**Figure 3.6:** The product ion spectrum of  $m/z$  457.7712  $[M+2H]$  for the selected signature peptide with sequence ANIQLVEK.

### 3.5 Discussion

Allergen identification and structure elucidation is a central aspect of any proteomic-based analysis of food allergens. Generally, IgE antibody binding to food proteins is identified using sera from allergic patients. Based on the frequency and intensity of IgE binding, specific proteins are located and isolated from a whole crude extract and its identity is investigated using different analytical techniques. In allergy research, N-terminal protein sequencing and mass spectrometry are generally used for the identification of IgE binding proteins.<sup>10</sup> Several allergens from various sources have been identified using mass spectrometric approaches.<sup>20-25</sup> Mass spectrometric analysis of allergens requires a set of methodological steps involving protein digestion for generation of peptides, elucidation of these peptides for their sequence data and analysis using bioinformatic tools and protein databases.<sup>26</sup> Most commonly, databases such as SEQUEST and Mascot are used for this purpose. The generated peptides are identified based on their mass-to-charge ratio and the allergen is finally identified by matching the derived amino acid sequence to known proteins. Digestion of the allergenic protein in question is commonly performed prior to MS analysis. For the generating of allergen-specific peptides, various enzymes were tested such as trypsin, GluC V8 and AspN.

In this chapter, a general strategy was demonstrated to characterise the important allergenic proteins present in Black tiger prawn. Tropomyosin, myosin light chain, and arginine kinase were identified using PMF, and some of their highly abundant peptides were *de novo* sequenced using PFF. Tropomyosin as the major allergen was completely sequenced using different enzymatic digestion strategies, derivatisation protocols, and ion sources for mass spectrometry. The IgE binding properties of TM, MLC and AK were confirmed in this study by the immunoblotting of black tiger prawn extract with patient sera.

One of the main advantages of non-immunological analytical techniques such as MS over traditional antibody-based immunoassays is the reduced effects of matrix interference. This can be mainly attributed to the digestion of proteins into specific peptides which are stable over time and can be easily detected using MS. This feature of mass spectrometric approaches is especially useful

for detection and quantification of allergenic proteins in food samples or air samples where unwanted proteins can interfere in antibody based assays such as ELISA leading to non-specific results.<sup>27</sup> Various enzymes are available for protein digestion, but trypsin is most commonly used due to its known cleavage sites between arginine (R) and lysine (K).<sup>26</sup> Apart from protein identification, this approach is central to the quantification of allergens using specific peptides. Peptides sequences that are unique to particular allergens are termed as “signature peptides”.<sup>27, 28</sup> These peptides can be generated using enzymatic digestion of the allergens in a reproducible and consistent way, which help in achieving high resolving power and sensitivity thus allowing MS to detect trace amounts of allergens.<sup>26</sup>

For BTP TM quantification purposes, the selection rules for a recommended signature peptide were followed. The experimental data for the resultant peptides were evaluated by bioinformatic approaches to determine the PTM-free peptides. The nominated candidates were subjected to the BLAST test to acquire the highest scoring signature peptide. This peptide (ANIQLVEK) can be chemically synthesised (labelled and unlabelled forms) and used in further studies to develop a detection and quantification method for BTP TM in different environments.

Black tiger prawn tropomyosin was, for the first time, *de novo* sequenced using bottom-up tandem mass spectrometry. Multiple types of enzyme digestion, ion sources, and derivatisation protocols were used to cover the whole amino acid sequence. Furthermore, the mass spectra of the peptide mass fingerprinting (PMF) and the peptide fragment fingerprinting (PFF) were uploaded to the Mascot search engine, which reported the amino acid sequence of the target allergen as tropomyosin. The post-translational modification motifs of TM were evaluated theoretically using bioinformatics tools and by visually screening through the mass spectra.

In summary, a novel robust chemical approach was established for the identification of novel allergenic proteins in black tiger prawns using mass spectrometry. Using this technique, three prawn IgE binding proteins were identified. Using a bottom-up approach, tropomyosin was sequenced and the

data published in the Genbank database. One signature peptide was designed and tested for Black tiger prawn tropomyosin, which was eight amino acids in length. This peptide will support the future development of analytical methods for the quantification of tropomyosin in food samples or air-borne tropomyosin in air samples from seafood processing factories.

Current *in vitro* IgE diagnostic methods rely on crude shellfish extracts (raw or boiled) for IgE quantification in the allergic patient's serum. There is a lack of understanding on the effects of thermal treatment on the allergen-IgE interactions. Having identified two novel allergens from Black tiger prawn, the aim of the next chapter was to identify the IgE binding proteins and characterise the prawn allergen repertoire using established mass spectrometric methods. The effects of heat processing of Black tiger prawns on IgE binding patterns of prawn allergens were investigated. MS methods developed in this chapter were used to identify novel putative allergens in raw and heat treated prawn extracts.

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### 3.7 Chapter 3 summary:

- The aim of this study was to establish mass spectrometric approaches for the identification of black tiger prawn allergens
- IgE binding was observed to several proteins, which were identified as tropomyosin, arginine kinase and myosin light chain
- Black tiger prawn tropomyosin was for the first time de-novo sequenced using a bottom-up MS approach
- The signature peptide for prawn tropomyosin was identified and validated, which will be a useful tool for the sensitive detection and quantification of this allergen in food matrices. The peptide sequence was “ANIQLVEK” located near the N-terminal region of tropomyosin, amino acid residue 67-74.

## CHAPTER 4

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### EFFECT OF HEAT PROCESSING ON ANTIBODY REACTIVITY TO ALLERGEN VARIANTS AND FRAGMENTS OF BLACK TIGER PRAWN – A COMPREHENSIVE ALLERGENOMIC APPROACH

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## 4.1 Introduction

Food allergy is based on the generation of IgE antibodies against particular proteins, i.e. allergens, which bind to FcεRI receptors on the surface of mast cells and basophils. Subsequent binding of allergenic proteins to these cell-bound IgE antibodies results in cross-linking of receptor-IgE complexes, which triggers the activation of the cells, resulting in mediator release and clinical symptoms. Current *in-vitro* diagnostics for allergy are based on the quantification of these specific IgE antibodies against various allergen extracts. Currently over 2,700 allergenic proteins have been identified, 20% of which are derived from food sources.<sup>1</sup> Food allergens can be classified into 71 protein families and these comprise less than 2% of all the known protein families. Allergenic proteins have specific biological functions; however the factors that make a protein allergenic are largely unknown.<sup>2</sup>

In Chapters 2 and 3 it was shown that the major allergenic protein in crustaceans currently characterised is tropomyosin (TM), a heat stable muscle protein that constitutes up to 20% of the total protein content.<sup>3-6</sup> TM has a highly conserved alpha helical coiled-coil structure and is reported to cause IgE cross-reactivity in patients with crustacean allergy to other arthropods such as house dust-mites.<sup>7</sup> Recent reports suggest that IgE, reactive to TM, may be a good marker for detection of clinical prawn allergy.<sup>8, 9</sup> However, the effects of food processing on the IgE binding reactivity to different shellfish allergens are poorly characterised at the molecular level. Moreover, it is unknown how food processing affects the detection of shellfish allergens using antibody-based tests, as required for food labelling regulations. Most studies on the molecular characterisation of crustacean allergens along with associated allergy prevalence studies mainly focus on single allergens and not on the complete allergen repertoire affected by food processing. There is a need to investigate the effects of heat treatment on the stability and IgE reactivity of different crustacean allergens present in whole extracts which may affect the diagnosis and detection of allergen-specific IgE in sensitised patients.

In Chapter 3, advanced mass spectrometric techniques were established for the detection and identification of IgE binding proteins in prawns. The aim of the

current chapter was to analyse the impact of heat processing on a comprehensive panel of prawn allergens, by utilising an allergenomic approach including patient IgE binding diversity and advanced mass spectroscopy. Since prawn allergens, which are clinically relevant to the Asia-Pacific region, are not fully identified, the Black tiger prawn (*Penaeus monodon*) was selected as a model species for analysis in this study due to its high consumption in the region.

## 4.2 Aims

The aims of this study were as follows,

- 1) To identify the IgE binding prawn allergens using sera from 16 adult patients with confirmed reactivity to prawns (*Penaeus monodon*)
- 2) To identify the prawn allergens using advanced mass spectrometry
- 3) To evaluate the effects of heat treatment on the IgE binding patterns of tropomyosin and other identified prawn allergens

## 4.3 Materials and Methods

### 4.3.1 Prawn heat treatment and protein extract preparation

The outer shell of fresh prawns (*Penaeus monodon*) was removed, the abdominal muscles shredded into small pieces and homogenised in 200 mL of phosphate buffered saline (PBS). This slurry was mixed for 3 hours at 4°C, followed by centrifugation at 14,000 rpm for 15 minutes. The supernatant was filtered through a glass-fibre filter, followed by a 0.45 µm membrane filter (Millipore, Billerica, MA, USA) and stored at -80°C until further use, named “P-raw” extract. An aliquot of the P-raw extract was heated at 100°C for 20 minutes in a water bath and the resultant extract centrifuged and filtered as above, named “P-H” extract. The third method, based on the natural exposure to crustacean allergens included heating the whole raw prawn with its outer shell in PBS at 100°C for 20 minutes and extracting the proteins as described above and named “P-WH”. The protein content of each prepared extract was calculated using the Bradford’s protein assay (1976).

### 4.3.2 Purification of natural TM

Tropomyosin was purified from the heated whole black tiger prawn using strong anion exchange chromatography as described in chapter 2. Briefly, 20 mg of the P-WH extract was loaded onto a Mini Macroprep High Q column (BioRad, Hercules, CA, USA) using 30 mM Tris-HCl buffer pH 6.8 as the mobile phase. TM was eluted using a gradient salt concentration from 400 mM to 600 mM of sodium chloride in the same mobile phase. The resultant TM fraction was dialysed overnight against PBS and stored at -80°C until further use.

### 4.3.3 Sequencing and expression of Black tiger prawn TM

Black tiger prawn tropomyosin was expressed as a recombinant protein, using the protocol detailed in Section 2.3.8 and 2.3.9. Briefly, total RNA was extracted from Black tiger prawns using the RNeasy RNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Single stranded cDNA

was generated by RT-PCR using a Transcriptor High Fidelity cDNA synthesis kit (Roche, Basel, Switzerland). The generated cDNA was used as a template to amplify the coding region of TM using forward (5'-GCGGATCC-GACGCCATCAAGAAGAAGATGC-3') and reverse (5'-GCGAATTC-TTAGTAGCCAGACAGTTCGCTG-3') primers and cloned into a sequencing vector, pCR 2.1 using the TOPO TA cloning Kit (Invitrogen, Carlsbad, CA, USA).

For recombinant TM expression, the coding region for TM was sub-cloned into the expression vector pRSET A (Invitrogen, Carlsbad, CA, USA) and transformed into BL21 *E.coli* cells using electroporation. Expression was induced using 0.6 mM IPTG from a fresh overnight culture in Luria broth containing 100 ug/mL ampicillin. Recombinant tropomyosin from Black tiger prawn (rPen m1) was subsequently purified from the crude cell lysate using nickel charged metal chelate affinity chromatography, dialysed in PBS and stored at -80°C until further use.

#### 4.3.4 Patients

Sixteen subjects with a convincing clinical history of allergic reactivity to shellfish and one non-atopic subject were recruited by The Alfred Hospital, Allergy Clinic, Melbourne, Victoria, Australia. Shrimp specific IgE antibodies were quantified using the ImmunoCAP system (Thermo Scientific, USA). Skin prick testing and oral challenge with crustacean extracts were not conducted routinely in these patients, in keeping with the clinicians' preference for safer serum specific IgE testing and use of clinical history of reactions on exposure. Demographic details of patients are given in Table 4.1. Ethics approval for this study was granted by James Cook University's Ethics committee (Project number H4313) in collaboration with The Alfred Hospital (Project number 192/07) and Monash University's Ethics Committees (MUHREC CF08/0225).

**Table 4.1:** Demographics of 16 shellfish allergic patients and one non-atopic control donor. Total IgE and shrimp specific IgE (f24) were quantified using ImmunoCAP®.

Patient	Sex	Age	Total IgE (kU/l)	Shrimp Specific (f24) IgE (kU/l)	Shellfish exposure	Symptoms	Reactivity to other reported seafood species	Reactivity to HDM	
								slgE (kU/L)	SPT (mm)
1	M	23	179	3.27	Oral	Laryngeal angioedema	Tuna, salmon, trout	NT	7 mm
2	F	24	372	1.82	Oral	OAS	-	3.90	9 mm
3	F	17	242	1.32	Contact	Asthma, Rhinitis	-	NT	7 mm
4	M	34	55	0.37	Oral	Asthma, angioedema, Itch	-	7.36	15 mm
5	M	43	136	4.54	Oral	OAS, Dyspnoea	Scallop	NT	NT
6	M	37	100	2.11	Oral	Asthma, Anaphylaxis, Urticaria	-	NT	NT
7	F	65	150	0.6	Oral	OAS, Asthma, Rhinitis	-	3.25	6 mm
8	M	51	164	0.4	Oral	Rhinitis, Hypotension, Erythema	-	NT	NT
9	F	56	440	0.5	Oral	Urticaria, OAS, Pruritus, Rhinitis	Crab	NT	5 mm
10	F	33	28	0.35	Contact	Urticaria, Rhinitis, Angioedema	Lobster	0.75	3 mm
11	M	23	158	0.85	Oral	Urticaria, Collapse, Dyspnoea, Angioedema	-	2.27	6 mm
12	M	46	822	5.33	Oral	Urticaria, Anaphylaxis	-	37.3	8 mm
13	F	20	3401	6.65	Oral	Asthma, Rhinitis, Urticaria, Anaphylaxis	-	>100	NT
14	M	21	283	19.7	Oral	Asthma, Urticaria, Angioedema, Rhinitis	-	51.3	NT
15	F	25	238	5.93	Oral	Anaphylaxis, Angioedema	-	16.70	8 mm
16	F	23	1946	9.5	Oral	Anaphylaxis, Angioedema, Rhinitis	Flounder, crab	14.10	NT
17 (control)	F	45	-	-	-	Non atopic	-	-	-

#### 4.3.5 SDS-PAGE and Immunoblotting

SDS-PAGE was performed as described previously in chapter 2 with modifications. Each extract (10 µg/well) was mixed with reducing sample buffer containing SDS and dithiothreitol, heated at 90°C for 3 min and loaded onto a 12% polyacrylamide gel. The protein components were resolved using a BioRad Mini-Protean Tetra cell (BioRad, Hercules, CA, USA) and stained using Coomassie brilliant blue R-250 to visualise the separated proteins.

For immunoblotting, the separated proteins were transferred to an activated PVDF membrane (BioRad, Hercules, CA, USA) and blocked with 5% skim milk powder (SMP) in PBS with 0.05% Tween-20 (PBS-T). For TM detection in various extracts, the proteins were exposed to monoclonal anti-TM antibody (Abcam, Cambridge, MA, USA) and subsequently rabbit anti-mouse IgG HRP labeled antibody (Sigma, USA). Antibody binding was visualised using the enhanced chemiluminescent technique (ECL).

To analyse the IgE binding patterns to prawn extracts, the membrane was blocked with 5% SMP in PBS-T and subsequently incubated overnight with 1:10 diluted patient sera using a slot blot apparatus (Idea Scientific, MN, USA). IgE binding was detected using HRP labelled rabbit anti-human IgE polyclonal antibody diluted 1:2500 (DAKO Corporation, USA) and visualised using the ECL method. Serum from a non-atopic donor was used as a negative control and a label control was included to confirm lack of reactivity by the secondary antibody to the extracts.

To characterise the IgE reactive protein band patterns, densitometric analysis was performed using the Geldoc System and Quantity One analytical software (BioRad, USA). Molecular weights of the antibody binding proteins were calculated against a standard curve of proteins with known molecular weight (BioRad, USA) using the Quantity One Software. Using the arbitrary density values of each patient's IgE binding protein bands, the antibody reactivity was graded as low, medium and strong.

For the analytical representation of IgE recognition patterns among the sixteen patient's to various prawn proteins, allergograms were created, based on IgE

reactive bands for each patient sera and their estimated molecular weights as calculated above. Analogous IgE-reactive protein bands among patients were grouped together with an error range of +/- 0.5 kDa.

#### **4.3.6 Mass spectrometric identification of IgE binding proteins**

The Black tiger prawn proteins reacting with patient serum IgE antibodies were excised from an equivalent SDS-gel for mass spectrometric analysis. The bands were de-stained and digested with trypsin as described previously in Chapter 3 using the DIONEX Ulti-242 Mate3000 Nano LC System (Germering, Germany). Tryptic peptides were separated on a nanoflow analytical column (LC Packing, Sunnyvale, CA) at 180 nL/min using a gradient regimen developed previously. Tandem spectra were searched using the NCBI Inr with the Matrix Science (Mascot) search engine (precursor and product ion mass tolerance set at 0.2 Da).

#### **4.3.7 Three dimensional homology modeling of identified prawn allergens**

Homology models of the identified prawn allergens were created using SWISS MODEL.<sup>10</sup> Three dimensional ribbon and space filling protein models were created using UCSF's Chimera software for visualisation of the allergenic protein's quaternary configuration.<sup>11</sup> For homology modelling, template protein structures were used for tropomyosin (PDB ID, 1C1G), arginine kinase (PDB ID, 4AM1), myosin light chain (PDB ID, 2W4A), sarcoplasmic calcium binding protein (PDB ID, 2SAS), fructose bisphosphate aldolase (PDB ID, 1FBA), triose phosphate isomerase (PDB ID, 2I9E) and titin (PDB ID, 1G1C).

#### **4.3.8 Cellular reactivity of prawn allergens**

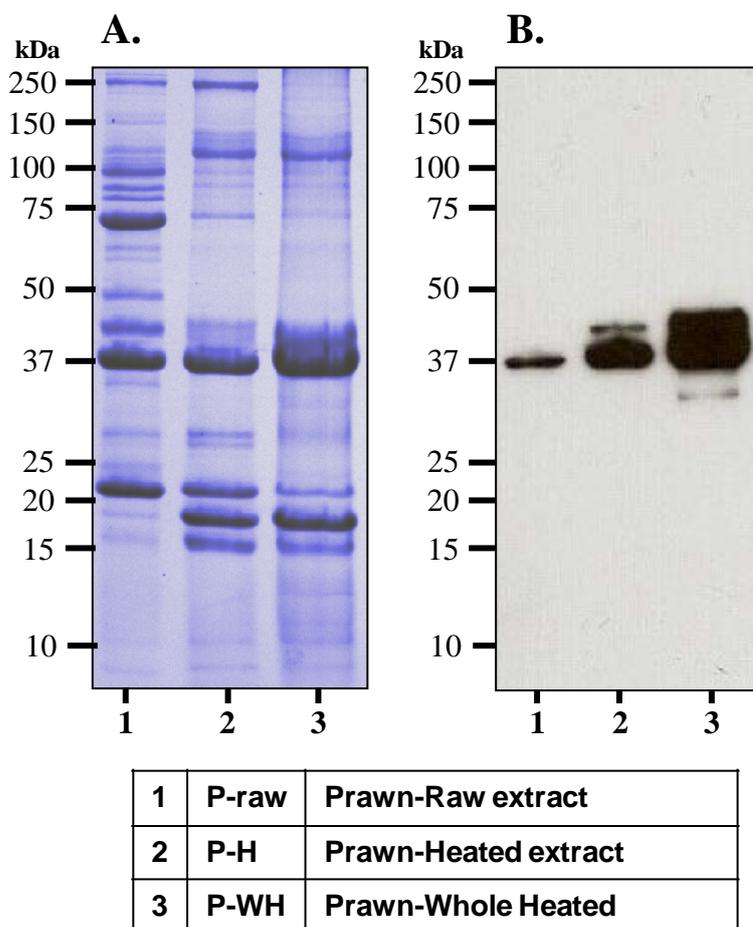
Confirmation of biologically relevant IgE reactivity of prawn allergens was demonstrated by activation of blood derived basophils as described

previously.<sup>12</sup> In brief the extracts were tested at increasing concentration (0.005 to 5 µg/ml) on basophils from whole blood of two prawn allergic patients, No. 2 and 12, having low and high shrimp specific IgE values, respectively. Activation of basophils were determined by up-regulation of CD63 surface expression on viable IgE<sup>hi</sup> cells (basophils) by flow cytometry. Positive controls included activation through rabbit anti-human IgE antibody (DAKO Corporation, Carpinteria, CA, USA) to induce IgE cross-linking and f-Met-Leu-Phe (Sigma, St. Louis, MO, USA) to induce IgE-independent activation of basophils. Stimulation buffer alone was used as a negative control. Results were expressed as the percentage of basophils loaded with IgE expressing high CD63 (IgE<sup>hi</sup> cells) for each concentration of allergen extract with the negative control level subtracted.

## 4.4 Results

### 4.4.1 Protein separation and allergen-specific antibody binding pattern

The heated extracts showed fewer protein bands compared to the raw extract, with major bands at about 37, 22, 18 and 16 kDa (Figure 4.1 A). The presence of TM in the analysed protein extracts was confirmed using a TM-specific monoclonal antibody (Figure 4.1 B). The antibody-binding pattern for TM in P-H was different compared to P-WH, indicating possible TM variants.



**Figure 4.1:** SDS-PAGE and immunoblot analysis of the prepared prawn extracts: (A) 12% SDS-PAGE of black tiger prawn extracts stained with Coomassie brilliant blue and (B) immunoblotting with monoclonal anti-TM antibody. 1) Raw muscle extract (P-raw), 2) muscle extract heat treated (P-H), 3) whole animal heat treated (P-WH).

#### 4.4.2 Patient demographics

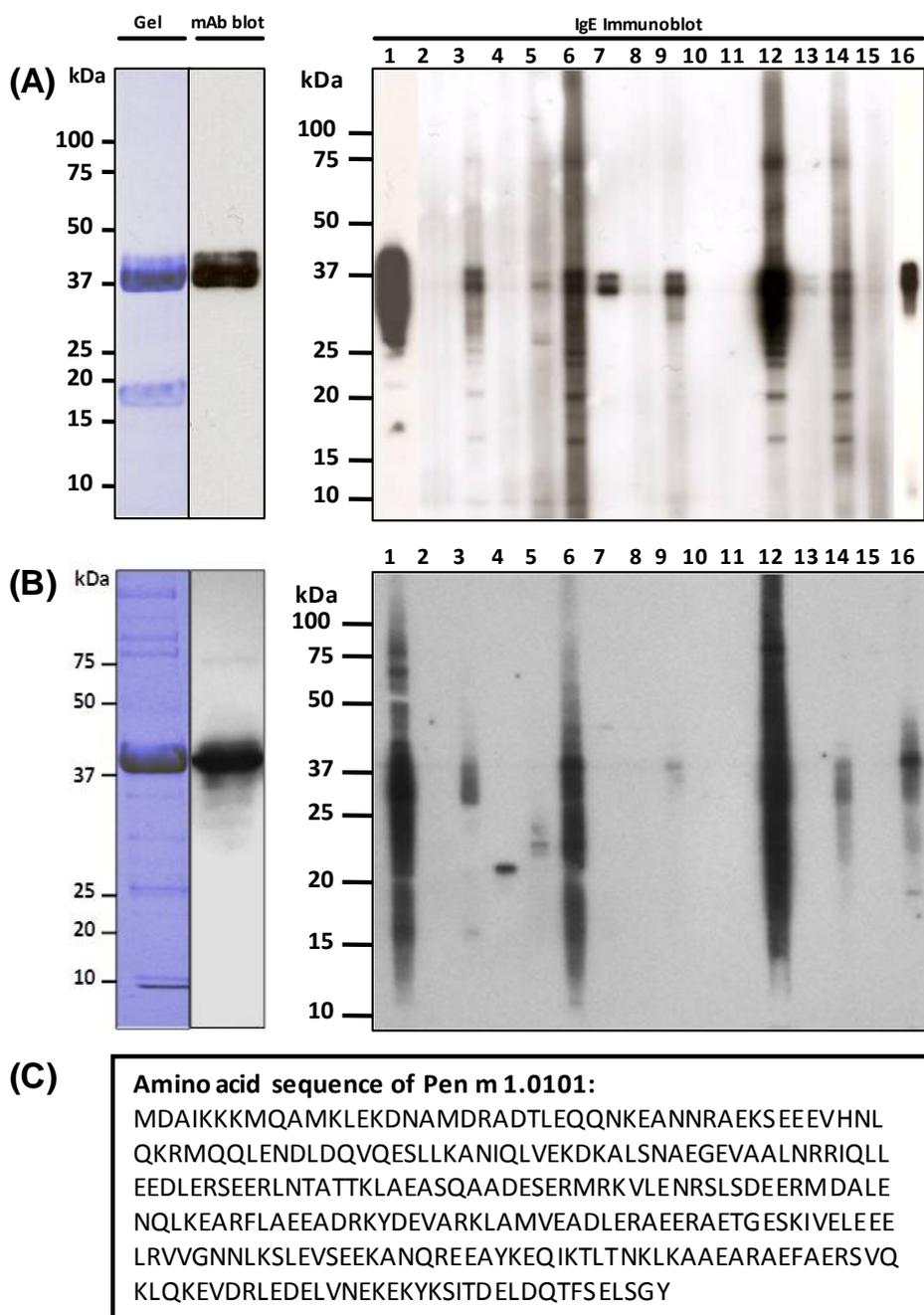
Eight female and eight male shellfish allergic patients, aged 17 to 65 years were recruited for the present study (Table 4.1). Total serum IgE levels ranged from 28-3401 kU/L and shrimp specific IgE ranged from 0.35 to 19.7 kU/L.

#### 4.4.3 Cloning and sequencing of Black tiger prawn tropomyosin

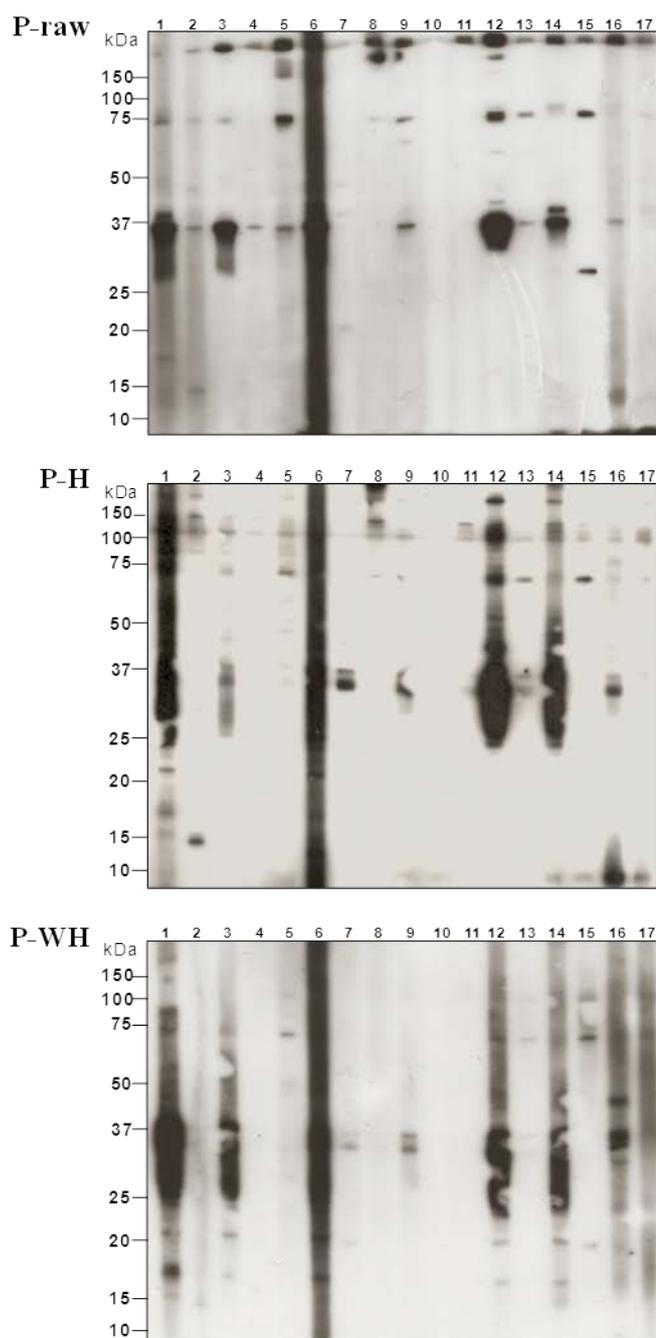
Black tiger prawn tropomyosin was generated as a recombinant protein in *E.coli* to analyse the IgE recognition pattern in patients to un-modified tropomyosin (Figure 4.2 A). Immunoblot analysis using monoclonal anti-TM antibody revealed a single band, confirming the presence of TM. Seven of the 16 patients (43%) showed strong IgE binding to the rPen m1 while 11 patients (69%) recognised the purified natural TM (Figure 4.2 A, 4.2 B). The coding region of TM was successfully amplified and the cDNA sequence (Pen m 1.0101) published in GenBank under the accession number HM486525.1 (Figure 4.2C).

#### 4.4.4 Patient IgE immunoblotting and allergogram analysis

Strong IgE binding was observed mainly for proteins in the range of 25-75 kDa for raw and heated prawn extracts (Figure 4.3). In order to compare and analyse the IgE antibody binding data from all the subject sera, allergograms were generated for each prawn extract (Figure 4.4). The MW of each IgE binding protein was calculated and IgE binding intensity of each band graded as low, medium or strong as detailed in the material and methods.



**Figure 4.2:** 12% SDS-PAGE Coomassie stain, monoclonal anti-TM antibody immunoblot and IgE immunoblot using sera from 16 shellfish allergic patients of (A) natural prawn tropomyosin, Pen m 1 and (B) recombinant prawn tropomyosin, rPen m 1. (C) Amino acid sequence of Pen m 1.0101 (Genbank accession number HM486525.1).

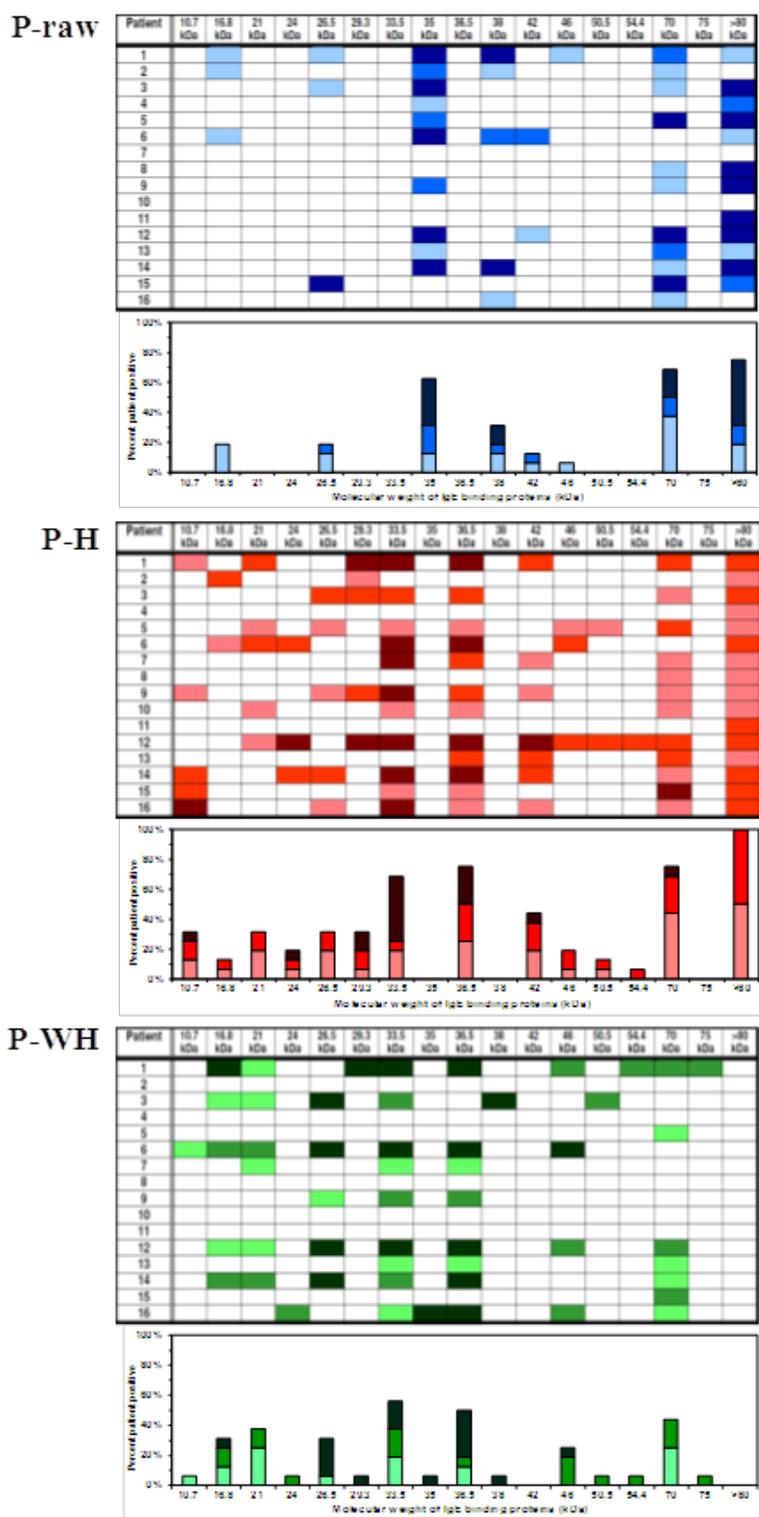


**Figure 4.3:** IgE antibody reactivity to black tiger prawn extracts with 16 shellfish allergic patient sera and one control non-atopic donor serum against raw muscle extract (P-raw), muscle extract heat treated (P-H) and whole animal heat treated (P-WH).

IgE binding to lower molecular weight proteins, between 10 kDa and 30 kDa, increased considerably after heating (Figure 4.4). High IgE binding was observed for a 35 kDa protein in P-raw, while heated extracts P-H and P-WH showed IgE binding to 33.5 and 36.5 kDa proteins in 50% of the patients. IgE reactivity was also noted to a 70 kDa protein. Eleven (68%) and seven (43%) patients demonstrated IgE binding to this protein in the raw and heated prawn extracts, respectively. A non-atopic control serum, lane 17, did not show significant binding to any of the prawn extracts or purified TM.

#### 4.4.5 Identification of IgE binding proteins by shotgun mass spectrometric analysis

Based on the IgE binding patterns of the prawn extracts, selected bands were excised from SDS-PAGE gels of P-raw and P-WH extracts for mass spectrometric identification (Table 4.2). TM was detected at 38.0 kDa in P-raw while it was identified at 33.5 and 36.5 kDa in P-WH. TM fragments were also identified at 26.5 kDa in P-WH (Appendix Table B 1.1). The eighteen peptides sequenced in this protein represent a large stable fragment of TM, excluding the N- and C- terminal ends of the complete protein structure (Appendix Figure B2.2). In addition, Sarcoplasmic calcium binding protein (SCBP), a recently identified allergen in Vannamei prawn (*Litopenaeus vannamei*), was detected at 26.5 kDa in P-raw as well as 16 kDa in P-WH. Arginine kinase (AK) was identified in P-raw at three different MWs: 42, 38 and 35 kDa. In contrast, AK was identified in P-WH at 36.5 kDa. Myosin light chain (MLC) was detected only in P-WH at 16 kDa but not in P-raw.



**Figure 4.4:** Allergogram analysis of patterns of IgE binding to proteins in different black tiger prawn extracts; P-row, P-H and P-WH. IgE binding intensities are graded as low, medium and strong, and percentage patient reactivity towards different IgE binding proteins is shown.

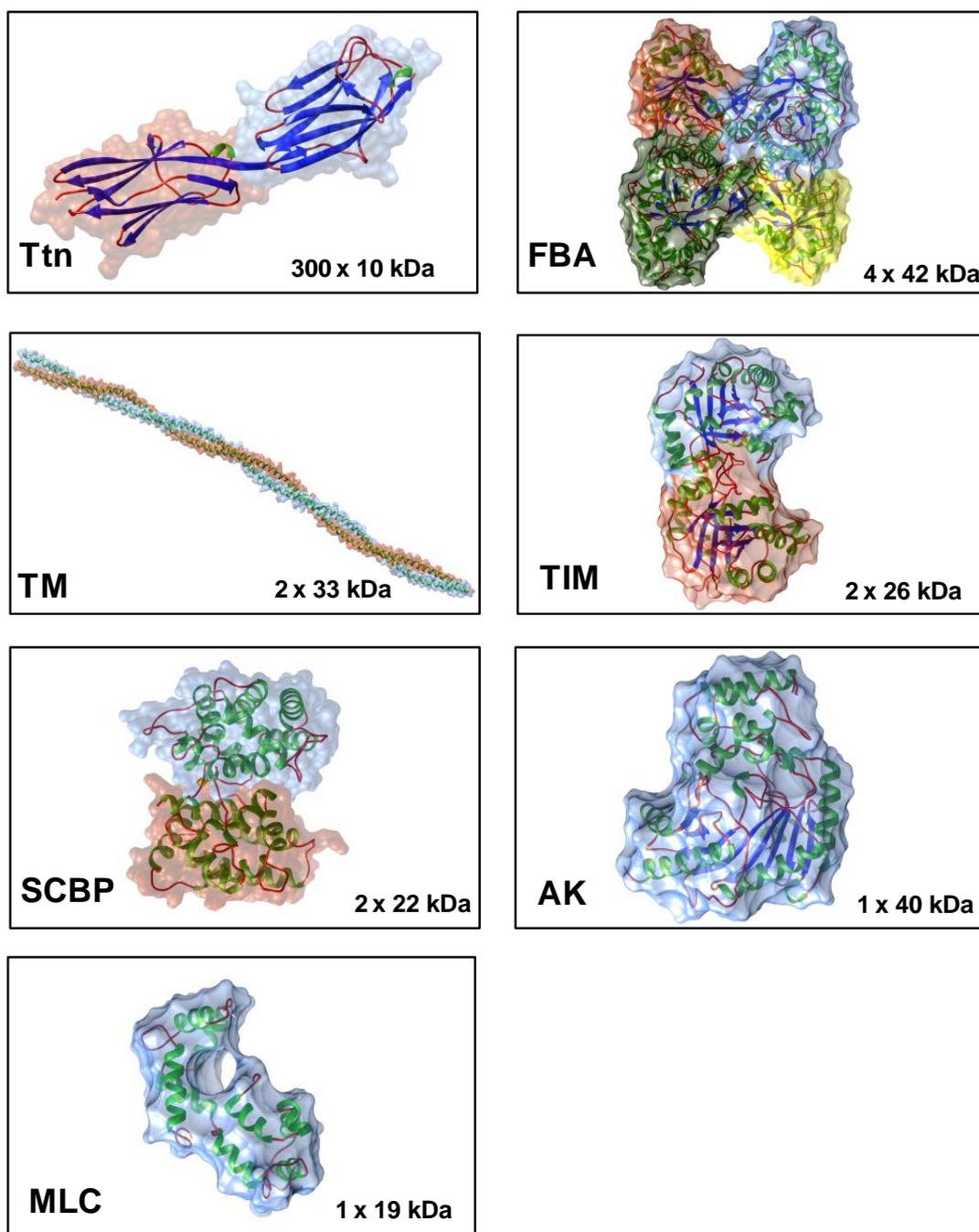
**Table 4.2:** IgE antibody reactive proteins identified by SDS-PAGE using LC-MS technique with sequence coverage denoted as percentage. IgE reactive proteins have been matched to known allergens using GenBank accession numbers. Number of patients tested positive with percentage in parentheses shown. See Appendix Table B 1.3 for full list of peptide sequences.

SDS-PAGE Band	Protein identity	Sequence coverage	Accession number (GenBank)	Protein function/ Mass, kDa	Known allergens	Patients tested positive (%) (n=16)
<b>Prawn-Raw (P-raw)</b>						
<b>70 kDa</b>	Titin fragment	2%	CAD60685.2	Muscle protein, 1200 kDa	-	11 (68%)
<b>42 kDa</b>	Arginine kinase	27%	GQ246164	Phosphotransferase, 40 kDa	Pen m 2	2 (12.5%)
	Putative fructose 1,6 bisphosphate aldolase	12%	Q9URB4	Glycolytic enzyme, 39.2 kDa	Cand a FBA	
<b>38.0 kDa</b>	Arginine kinase	29%	GQ246164	Phosphotransferase, 40 kDa	Pen m 2	5 (31%)
	Tropomyosin	15%	HM486525	Muscle protein, 37 kDa	Pen m 1	
<b>35 kDa</b>	Arginine kinase	37%	GQ246164	Phosphotransferase, 40 kDa	Pen m 2	10 (62%)
<b>26.5 kDa</b>	Sarcoplasmic calcium binding protein	39%	FJ184279	Muscle protein, 20 kDa	Lit v 4	3 (19%)
<b>16.8 kDa</b>	Triose-phosphate isomerase	8%	FJ462738	Isomerase enzyme, 28 kDa	Cra c 8	3 (19%)
<b>Prawn-Whole Heated (P-WH)</b>						
<b>70 kDa</b>	Titin fragment	2%	CAD60685.2	Muscle protein, 1200 kDa	-	7 (43%)
<b>38 kDa</b>	Tropomyosin	48%	HM486525	Muscle protein, 37 kDa	Pen m1	1 (6%)
<b>36.5 kDa</b>	Arginine kinase	11%	GQ246164	Phosphotransferase, 40 kDa	Penm 2	8 (50%)
	Tropomyosin	47%	HM486525	Muscle protein, 37 kDa	Pen m 1	
<b>33.5 kDa</b>	Tropomyosin	48%	HM486525	Muscle protein, 37 kDa	Pen m 1	9 (56%)
<b>26.5 kDa</b>	Tropomyosin fragment	10%	HM486525	Muscle protein, 37 kDa	Pen m 1	5 (31%)
<b>16.8 kDa</b>	Myosin light chain	9%	ADM34185	Muscle protein, 20 kDa	Lit v 3	5 (31%)
	Sarcoplasmic calcium binding protein	14%	FJ184279	Muscle protein, 20 kDa	Lit v 4	

For the first time the allergen fructose 1,6-bisphosphate aldolase (FBA), previously identified in cockroach<sup>13</sup>, was identified in Black tiger prawn at 42 kDa in P-raw but not in P-WH, consistent with heat sensitivity. Two additional novel allergenic proteins were identified in this prawn species. Triose-phosphate isomerase (TIM) previously identified in North Sea shrimp<sup>14</sup> was detected at 16 kDa in P-raw. Furthermore, a 70 kDa IgE binding fragment of the invertebrate muscle protein Titin (Ttn), one of the largest known proteins was identified with IgE binding in P-raw and P-WH, demonstrating heat stability.

#### 4.4.6 Homology modelling of identified allergens

3D homology models of the identified allergens were created for better visualisation of the allergen's tertiary and quaternary protein structure in their natural state (Figure 4.5). TM exists as a coiled-coil dimer made of two alpha-helical subunits, 33 kDa in size each. AK and MLC are present in a monomeric globular form of 40 kDa and 19 kDa, respectively. Similar to TM, SCBP and TIM form a dimer with subunits of 22 kDa and 26 kDa respectively. FBA forms a tetrameric structure of four identical subunits, each 42 kDa in size. In contrast, titin exists as a super repeat of Ig-like and fibronectin-like domains with a native size of around 3000 kDa. The biochemical and biological properties and functions of the identified allergens are summarized in Table 4.3. Five of the seven prawn allergens have a pI below 6, while FBA and AK have very basic pI values of 8.6 and 8.68, respectively.



**Figure 4.5:** Three dimensional homology structural models of allergenic proteins identified in Black tiger prawn. Titin's (Ttn) molecular structure consists of 300 tandem repeats of immunoglobulin-like and fibronectin-like domains. Triose-phosphate isomerase (TIM) forms a tetramer; Tropomyosin (Tm), Sarcoplasmic calcium binding protein (SCBP) and Fructose 1,6 bisphosphatealdolase (FBA) form a homo-dimer in their physiological state; Arginine kinase (AK) and Myosin light chain (MLC) are functional in their monomeric forms.

**Table 4.3:** Biochemical properties and physiological functions of identified black tiger prawn allergenic proteins.

Allergens	Native size (kDa)	Identified size of IgE bands (kDa)	Heat stability of allergen	Native conformation	Function	Route of exposure	pI value of allergen
<b>Ttn</b>	3000	70	Stable	Tandem repeats of immunoglobulin-like and fibronectin-like units	Passive muscle elasticity	Ingestion	4.96-5.24
<b>FBA</b>	39-43	42	Labile	Tetramer	Glycolysis (energy metabolism)	Ingestion Inhalation	5.69-8.68
<b>TM</b>	33-38	38, 33, 26	Stable	Dimer	Muscle contraction	Ingestion Inhalation	4.57-4.76
<b>SCBP</b>	22	16.8	Stable	Dimer	Muscle contraction regulation	Ingestion	4.60-5.06
<b>TIM</b>	26-29	16.8	Labile	Dimer	Glycolysis (energy metabolism)	Ingestion Inhalation	5.67-6.24
<b>AK</b>	39-40	42, 38, 35	Stable	Monomer	Energy for muscle contraction	Ingestion Inhalation	5.58-8.60
<b>MLC</b>	19-22	16.8	Stable	Monomer	Muscle contraction	Ingestion	4.23-4.67

#### 4.4.7 Selective recognition of Tropomyosin

Due to TM's major allergen status in shellfish allergy and wide usage in *in-vitro* diagnosis as well as detection in consumer products for food labelling, a comparative analysis was performed between the IgE binding patterns of the patients to natural un-treated or processed TM and the recombinant form of TM. Based on the IgE antibody binding patterns to natural and recombinant TM (rPen m1), as well as other IgE binding proteins by immunoblotting, subjects could be divided into Group A, with 'strong TM binding' (n=10) and Group B, with 'weak/no-TM binding' (n=6) (Table 4.4). All subjects of the 'strong TM binding' group exhibited mild or severe symptoms on exposure to shellfish, but only 70% of these patients also recognised rPen m1. These findings highlight the possible implication of post-translational protein modifications in the allergic reaction. In contrast, despite all six subjects in the 'weak/no TM binding' group having also mild to severe clinical reactions to shellfish, only 50% demonstrated weak binding to natural TM and none recognised rPen m1. However, 50% of the latter group demonstrated IgE binding to allergens other than TM, indicating the possible importance of non-TM allergens as the main allergen for some patients.

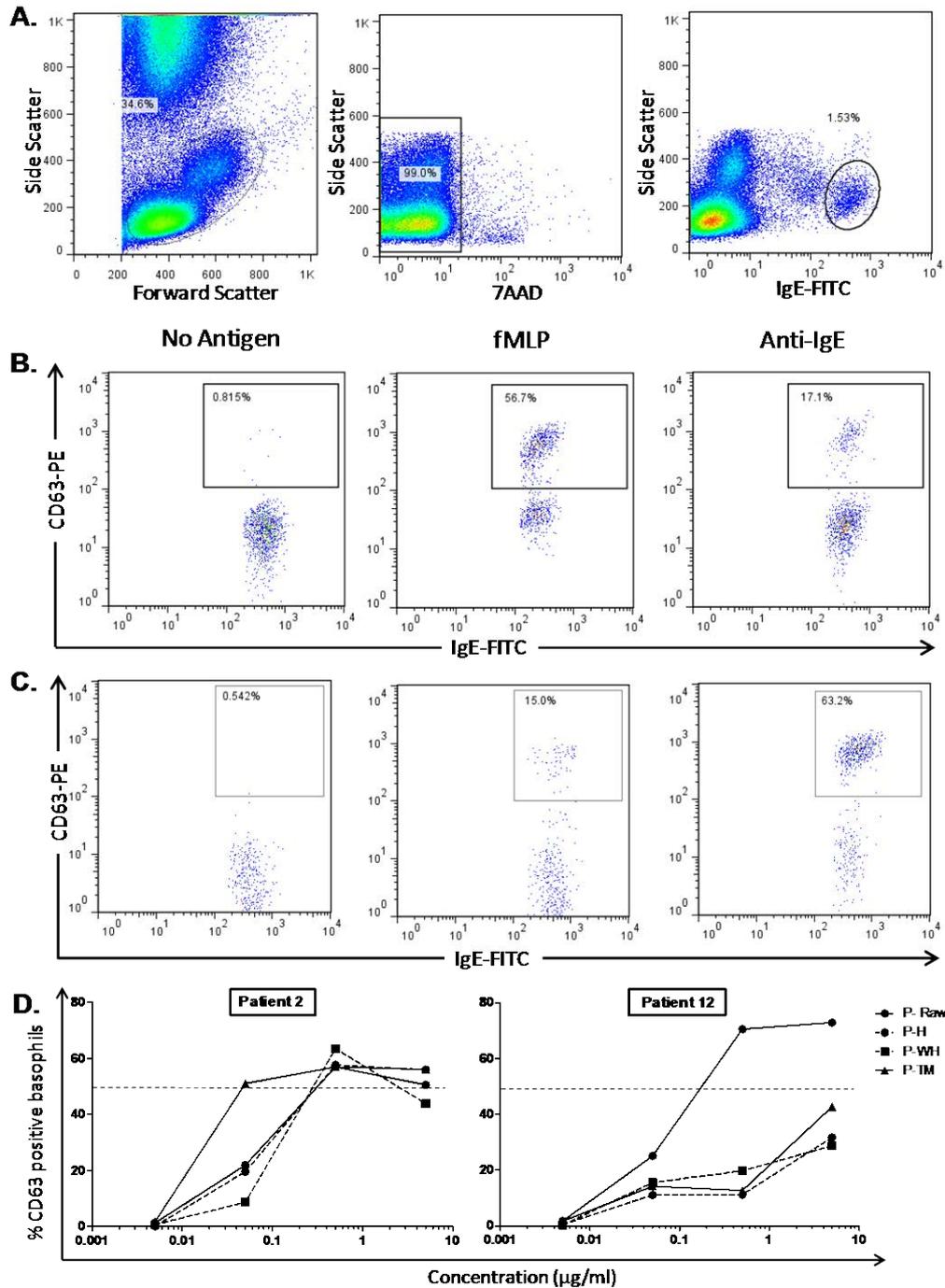
#### 4.4.8 Cellular reactivity of prawn allergens

To demonstrate biologically relevant IgE reactivity of the different prawn extracts, activation of isolated basophils from two patients were analysed. In a basophil activation assay, fresh blood is collected from the allergic patient. This contains sensitised basophils with allergen specific IgE on Fc $\epsilon$ RI receptors. These cells are exposed to different concentrations of the allergen extract (different prawn extracts) which initiates IgE cross-linking and expression of CD63 which is associated with intracellular vesicles and is a marker for cell degranulation. Representative data is shown in Figure 4.6. Patient 2 demonstrated higher basophil sensitivity (i.e. basophil activation by lower concentrations of extract) to natural prawn tropomyosin (P-TM; Figure 4.6, C) compared to the other Black tiger prawn extracts. In contrast, patient 12 showed markedly higher basophil activation to allergens from P-raw compared

to the heated prawn extracts (Figure 4.6, D). Patient 2 sera had a high shrimp-specific IgE count and showed high sensitivity to tropomyosin and the heated extract. This result was in concurrence with the immunoblotting data in which patient 12 demonstrated high IgE binding to natural and recombinant tropomyosin. In contrast, patient 2 with low shrimp specific IgE titre demonstrated higher basophil sensitivity to the raw extract as compared to tropomyosin and the heated extracts. These results demonstrated sensitivity and IgE reactivity to yet unknown heat-labile prawn allergens, but not to tropomyosin as supported by the immunoblotting data.

**Table 4.4:** IgE antibody binding reactivity of patient sera to natural and recombinant TM and other allergens in the raw and heated prawn extracts by immunoblotting. Symptoms have been grouped as severe or mild\*.

Patient	Total IgE (kU/l)	Shrimp Specific (f24) IgE (kU/l)	IgE binding to natural Tropomyosin	IgE binding to recombinant Tropomyosin	IgE binding to other allergens		Symptoms
					Raw extract	Heated extract	
<b>Group A: Patients with strong IgE antibody binding to tropomyosin</b>							
1	179	3.27	Yes	Yes	Yes	Yes	Mild
3	242	1.32	Yes	Yes	Yes	Yes	Severe
5	136	4.54	Yes	No	Yes	Yes	Mild
6	100	2.11	Yes	Yes	Yes	Yes	Severe
7	150	0.16	Yes	No	No	No	Severe
9	440	0.5	Yes	Yes	Yes	Yes	Mild
12	822	5.33	Yes	Yes	Yes	Yes	Severe
13	3401	6.65	Yes	No	Yes	Yes	Severe
14	283	19.7	Yes	Yes	Yes	Yes	Severe
16	1946	9.5	Yes	Yes	Yes	Yes	Severe
<b>Group B: Patients with weak/no IgE antibody binding to tropomyosin</b>							
2	372	1.82	No	No	Yes	No	Mild
4	55	0.37	Low	No	No	No	Severe
8	164	0.04	Low	No	Yes	No	Mild
10	28	0.21	No	No	No	No	Mild
11	158	0.85	No	No	No	No	Severe
15	238	5.93	Low	No	Yes	Yes	Severe



**Figure 4.6:** Activation of basophils were determined by up-regulation of the CD63 cell surface marker on viable high IgE binding cells (IgE hi cells, basophils) by flow cytometry. (A) and expression of cell surface CD63 in negative and positive controls (B) and with increasing concentration of prawn extract (C). D) Dose response to different prawn extracts for two subjects (No. 2 and 12). Basophils were stimulated with P-raw (●), P-H (●, dotted line), P-WH (■, dotted line), P-TM, natural prawn tropomyosin (▲) and shown as percentage of CD63 positive basophils.

## 4.5 Discussion

Shellfish allergy is one of the primary causes of hypersensitivity reactions to food.<sup>15</sup> The current *in-vitro* diagnostics for shellfish allergy are based on the quantification of allergen-specific antibodies. Although TM is accepted to be the main shellfish allergen, crude sub standardised preparations of prawns (raw or boiled) are generally employed in most of the current *in-vitro* and *in-vivo* diagnostics. Component resolved diagnosis for prawn allergy makes use of only prawn TM (Pen a1) for specific IgE quantification, and other identified allergens are not taken into account. Similarly, to comply with food labelling regulations (Food Allergen Labelling and Consumer Protection Act of 2004), immunoassays commonly used to detect shellfish allergens in processed food, often utilise antibodies raised against uncharacterised crude extracts, or detect only TM as a representative shellfish allergen. Thus, other identified shellfish allergens may often be overlooked and need to be investigated in further detail. Novel crustacean allergens have been identified in recent years. However, there is still a lack of a comprehensive allergenomic analysis of the whole allergen repertoire of prawns.

In the present study, IgE-reactive prawn proteins were identified and characterised and further analysed for modification in reactivity due to the “cooking” process, using patient IgE screening and mass spectrometric analysis. Additionally three putative novel prawn allergens were identified. Patients showed different reactivity to these allergens, but importantly, of the patients who did react with these allergens, not all were TM reactive. This indicates that these new allergens as well as TM should be included in a more detailed component resolved diagnostic approach for shrimp allergy. It was also demonstrated that heating of whole prawns increases the IgE reactivity of specific allergenic proteins.

TM is a highly cross-reactive pan-allergen among crustaceans, insects and dust-mites.<sup>7, 15</sup> Prawn TM-specific IgE has been suggested to be the best single molecular marker for predicting clinical symptoms of crustacean allergy.<sup>8, 9</sup> Specificity of IgE antibody to TM has been shown to be around 70%, which

suggests that it may not lead to accurate diagnosis of general crustacean allergy, and other allergens might be of importance.<sup>16</sup>

Impact of heat processing on shellfish allergenicity has been discussed previously. Recent studies conducted with patients allergic to blue swimmer crab from the Asia-Pacific region demonstrated differential IgE reactivity to raw or cooked allergen extracts. Cellular activation experiments in the current study support these findings with different antibody reactivity to heat-stable allergens, such as TM, and possible reactivity to heat-labile allergens including FBA and TIM.

Using allergogram analysis of the IgE reactivity it was observed that 30% of patients recognised TM in the raw extract. Heating of the extract caused an increase in this IgE reactivity where 87% of patients reacted to three different TM variants. The additional higher MW TMs are most likely generated during the heating process, which demonstrated increased antibody binding after heating, suggesting conformational changes which may have led to better epitope recognition.<sup>17</sup> The smaller 26.5 kDa TM variant was characterised in chapter 2 as being a stable peptide fragment of the native TM.<sup>18</sup>

In addition to TM, yet another crustacean allergen which also showed 62% of patient recognition was identified as AK. Unlike TM, AK showed presence of three distinct antibody binding variants in the raw extract. Interestingly, after heat treatment AK-specific IgE reactivity was reduced to just one AK variant at 36.5 kDa. A recent study demonstrated that the heat-stable novel AK from Northern shrimp can be aerosolised and cause inhalational sensitisation in the seafood workplace.<sup>19</sup>

Sarcoplasmic calcium binding protein and myosin light chain, two additional allergens, previously identified in other prawn species<sup>20, 21</sup>, were both recognised by 30% of the patients involved in this study. However, IgE binding to MLC was only observed in the heated prawn extract and not in raw. In contrast, a study by Ayuso *et al* demonstrated stronger IgE binding to Lit v 3 in raw extract, however only in the adult population.<sup>20</sup> The different IgE reactivity in this study may indicate different expression levels of MLC in Black tiger prawn in addition to presence of possible isoforms and the impact of heat

treatment. While MLC has not been analysed in the literature as detailed as TM, this study confirms the heat stability of MLC also in Black tiger prawn.

The current study identified three novel allergens, not previously characterised in Black tiger prawns, of which titin could have the 'major allergen' status according to the IUIS guidelines.<sup>22</sup> Titin, a 3000 kDa protein is one of the largest known proteins<sup>23</sup> and is responsible for passive muscle elasticity. The secondary structure of titin consists of tandem repeats of immunoglobulin-like and fibronectin-like domains. We have identified a heat-stable 70 kDa IgE-reactive fragment of this protein, which belongs to the immunoglobulin-like domain, and is highly conserved among invertebrates (e.g. crayfish, oyster, silk worm).<sup>24, 25</sup> 68% of the patients showed reactivity to this protein fragment. Fructose 1,6 bis-phosphate aldolase (FBA) is an approximately 160 kDa enzyme, which exists in a tetrameric form and is involved in glucose energy metabolism. FBA was previously characterised as an allergen in various fish species and nematode parasites (*Anisakis*). We have for the first time identified FBA as an IgE reactive protein in a crustacean species which is a substantial find for improved diagnosis and management of shellfish allergy. Triose-phosphate isomerase (TIM) is a 26-29 kDa enzyme protein which exists as a dimer, and similar to FBA is involved in glucose energy metabolism. This allergen was previously identified in Northern shrimp (*Crangon crangon*) and cockroach (*Blattella germanica*).<sup>13, 14</sup> The identification of IgE-reactive TIM in Black tiger prawn highlights its importance as a possible invertebrate pan allergen.

Most of the identified Black tiger prawn allergens (5/7) in the current study exist in their native configuration as dimers or oligomers. The formation of oligomeric protein aggregates seems to have considerable influence on the allergenic reactivity as discussed by Rosenberg.<sup>26</sup> Highly arrayed structures as found on large non-denatured protein aggregates, are potent inducers of antibody responses. In the case of allergenic proteins, this might relate to the ability of multivalent allergens to extensively cross-link IgE-receptor complexes on B-cells as well as mast cells and basophils. The formation of dimeric and oligomeric allergens seems to be more common than expected.<sup>27</sup> A recent study showed presence of homo-dimers in over 80% of the protein crystal

structures of 55 different food or inhalant allergens analysed. Also hypoallergenic variant of the major milk allergen Bos d 5 was shown to exist in its native state as a monomer.<sup>27</sup> Of the seven prawn allergens identified in this study, TM, TIM and SCBP exist as dimers, FBA as a tetramer and Ttn is essentially a super-repeat of similar structured domains. The quaternary structure of the allergenic protein may offer enhanced stability to the molecule thereby increasing its IgE reactivity by helping in efficient cross-linking by the antibody. Current diagnostics for shellfish allergy may not be able to detect IgE antibodies targeted to oligomeric allergens. Moreover, specific IgE to these oligomers might vary among different patients.

The findings of this study will help towards better standardisation of allergen extracts used in *in-vitro* diagnostics and towards improved detection of shellfish allergens in processed food products to protect sensitised consumers.

Residual IgE reactivity to allergen fragments generated by enzymatic digestion of TM has been previously demonstrated.<sup>28</sup> The presence of natural and heat-induced IgE-reactive fragments of TM, Ttn, AK, TIM and MLC was reported in this study. While allergen fragments of Ttn, AK and TIM may occur due to natural factors, the current findings suggest that the TM and MLC fragments could also be generated due to thermal processing. These findings are supported by analysis of a stable TM fragment in Chapter 2, comprising of five out of eight previously characterised linear IgE binding epitopes<sup>29</sup> generated during heat processing.<sup>18</sup>

In summary, heating causes a considerable increase in IgE reactivity to prawn allergen extracts. In addition to TM, AK, MLC and SCBP, we have identified three novel allergens, Ttn, FBA and TIM in Black tiger prawns. Further studies using recombinant protein expression and analysis is required to confirm clinical reactivity of these novel prawn allergens. Allergen fragments, in addition to the native allergens are possible inducers of IgE reactivity in patients. Most of the identified prawn allergens exist in multimeric configurations which may have implications on current allergy diagnostics and allergen detection in food sources. The outcomes of this study expands the current knowledge on

allergenic protein-IgE interactions and will facilitate improved allergen detection and development of novel diagnostic strategies.

In the next chapter, the differential IgE binding to tropomyosin from raw and heat treated extracts of two commonly consumed prawn species is compared using a cohort of prawn allergic patients. In addition, the allergen tropomyosin of King prawn is sequenced and molecular and immunological characteristics compared to tropomyosin from Black tiger prawn.

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#### 4.7 Chapter 4 summary

- Alterations in the IgE-antibody reactivity to prawn allergens due to thermal processing are not fully understood. The aim of this study was to analyse the impact of heating on prawn allergens using a comprehensive allergenomic approach.
- IgE antibody binding proteins were identified by advanced mass spectroscopy, characterised by molecular structure analysis and their IgE reactivity compared among the prepared black tiger prawn extracts.
- Allergens identified were tropomyosin, arginine kinase, myosin light chain, sarcoplasmic calcium binding protein, and putative novel allergens including triose phosphate isomerase, fructose bis-phosphate aldolase, and titin.
- The prawn allergens were mostly heat-stable and formed dimers or oligomers. Thermal treatment enhanced antibody reactivity to prawn allergens as well as fragments and should be considered in the diagnosis of prawn allergy and detection of crustacean allergens in processed food.

## CHAPTER 5

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DIFFERENTIAL IGE REACTIVITY TO BLACK TIGER PRAWN (*PENAEUS MONODOM*) AND KING PRAWN (*MELICERTUS LATISULCATUS*)

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## 5.1 Introduction

The term shellfish comprises of a wide range of species. According to the biological classification, shellfish can be simply divided into crustaceans and molluscs from the phylum arthropoda and phylum mollusc, respectively. Nearly, 30,000 crustacean species and nearly 100,000 mollusc species have been identified worldwide. Among crustaceans, nearly 40 different prawn species have been identified. Due to the proximity in the biological evolution, the major allergens found in these species are also closely related. This has led to the occurrence of clinical cross-reactivity in allergic patients on exposure to various invertebrate allergens. Clinical cross-reactivity can occur when IgE antibodies produced against a specific allergen is able to bind to a closely related allergen, which may be from another source or species, and eventually result in IgE cross-linking on basophils and mast cells leading to mediator release and clinical symptoms.

Clinical cross-reactivity is often observed among food and inhalant allergens. For example, the Birch pollen allergen, Bet v1 has been shown to be cross-reactive to carrot allergen, *Cor a1*.<sup>1</sup> In the case of peanut allergy, cross-reactivity has been demonstrated between *Ara h2* and non-homologous allergens, *Ara h1* and *Ara h3*.<sup>2</sup> Cross-reactivity has also been demonstrated between lupins and peanut allergens in children.<sup>3</sup>

Clinical cross-reactivity has also been reported among allergens from various invertebrate species. A study by Goetz and Whisman confirmed the cross-reactivity between shrimp and scallop in a seafood restaurant worker.<sup>4</sup> Orthodox jews unexposed to shrimps have been demonstrated to elicit IgE antibody reactivity to shrimps due to house dust mite sensitisation.<sup>5</sup> Cross-reactivity has also been demonstrated among storage mites, dust mites and shrimps.<sup>6</sup> Moreover, cross-sensitisation has been demonstrated between mites, cockroaches and shrimps.<sup>6-9</sup>

As demonstrated in Chapter 2, Tropomyosin is the major invertebrate allergen found in a wide range of invertebrate species and displays a high conservation in the primary structure. Several studies have shown the significance of tropomyosin in IgE antibody cross-reactivity among various invertebrate

species. Shrimp allergic patient IgE antibody was shown to bind to cockroach and dust mite tropomyosin.<sup>8, 10</sup> In another case, immunological cross-reactivity has been demonstrated between filarial and mite tropomyosin and between ascaris and cockroach.<sup>11, 12</sup>

Previous clinical studies have indicated that the serum IgE antibody repertoire differs in allergic patients. This may be due to various factors such as allergen exposure and sensitisation to specific sources e.g. food or inhalant allergenic source. Depending on these factors, sensitised patients may elicit clinical symptoms to multiple sources, e.g. shrimps, molluscs and dust mites or only to a single group, e.g. crustaceans such as prawns, crabs and lobsters. Several studies have attempted to analyse the significance of tropomyosin as a marker of shellfish allergy. For example, a recent study in 2011 has shown tropomyosin to be a good predictor of shrimp allergy with a positive predictive value of 0.72 and negative predictive value of 0.91.<sup>13</sup> Moreover, tropomyosin is the only shellfish allergen available in commercial allergy diagnostic platforms for component testing of shellfish allergy. Although studies have been performed to analyse the clinical and molecular cross-reactivity of tropomyosin between various species, the individual patient IgE antibody binding characteristics to tropomyosin from closely related species has not been investigated in detail.

A study in 2002 elucidated the IgE binding epitopes on *Pen a 1* from Brown shrimp (*Penaeus aztecus*).<sup>14</sup> It was shown that, different patients elicited IgE antibody binding to the TM epitopes at different intensities and frequency.

In the previous study (Chapter 4), differential patient IgE binding was demonstrated to various Black tiger prawn tropomyosin in 16 shellfish allergic patients. This highlighted the importance of allergen primary structure and patient IgE antibody repertoire in deciding the IgE cross-reactivity to various allergenic sources. However, there is still a lack of information on the conservation or variability of the amino acid sequence specific to the IgE epitopes on tropomyosin from a wide range of invertebrate species.

The aim of this study was to analyse the differential IgE antibody binding to tropomyosin (TM) from two closely related prawn species and investigate the molecular basis of IgE cross-reactivity. Furthermore, the second aim was to

compare the amino acid sequences of tropomyosins from an extensive panel of invertebrate species, specific only to the previously identified eight IgE binding regions. In this chapter, tropomyosin was identified from King prawns, a commonly consumed species in Australia and its IgE antibody binding was compared to Black tiger prawn tropomyosin using *in vitro* IgE binding assays. Different raw and heated prawn extracts were compared for their IgE reactivity and clinical relevance using patient basophil activation. A comprehensive amino acid sequence comparison was performed for different antibody binding regions of tropomyosin to analyse their degree of conservation among different species.

## 5.2 Aims

The specific aims for the work described in this chapter were

- To identify and characterise tropomyosin (TM) from the commonly consumed King prawn (*Melicertus latisulcatus*) using IgE immunoblotting, mass spectrometry and cDNA sequencing
- To evaluate the differential IgE binding patterns to tropomyosin from two prawn species; Black tiger prawn (*Penaeus monodon*) and King prawn (*Melicertus latisulcatus*) and investigate the effects of heat-processing on the differential IgE binding
- To analyse and compare the primary amino acid sequence of IgE epitopes of tropomyosin in a wide range of invertebrate species to investigate the molecular basis of IgE cross-reactivity

## 5.3 Methods

### 5.3.1 Protein extraction

Protein extraction was performed as previously described in Chapter 4. The outer shell of fresh king prawns (*Melicertus latisulcatus*) (KP) and Black tiger prawns (*Penaeus monodon*) (BTP) were removed and the abdominal muscles shredded into small pieces and homogenised in 200mL of phosphate buffered saline (PBS). This slurry was mixed over night at 4°C followed by centrifugation at 14,000 rpm for 15 minutes. The supernatant was filtered through a glass-fiber filter followed by 0.45µm membrane filter (Millipore, Billerica, MA, USA) and stored at - 80°C until further use, named “raw-extract”. An aliquot of the “raw-extract” was heated at 100°C for 20 minutes in a water bath and the resultant extract centrifuged and filtered as above, named “HE1 extract”. The third method, based on the natural exposure to crustacean allergens included heating the whole raw prawn with its outer shell in PBS at 100°C for 20 minutes, named “HE2 extract”. The protein concentration of different extracts was verified using Bradford assay.

### 5.3.2 Prawn allergic subjects

Ten subjects with a clinical history of reactivity to shellfish and one non-atopic subject were recruited by The Alfred Hospital, Allergy Clinic, Melbourne, Victoria, Australia. Prawn specific IgE antibodies were quantified using the ImmunoCAP system (Thermo Scientific, USA). Demographic details of patients are given in Appendix Table B 1.4. Ethics approval for this study was granted by The Alfred Hospital (Project number 192/07) and Monash University (MUHREC CF08/0225) Ethics Committees.

### 5.3.3 SDS-PAGE

SDS-PAGE was performed as described in Chapter 2, Section 2.3.4.

### 5.3.4 Immunoblotting (mAb and IgE) and allergogram

mAb and IgE immunoblotting using subject sera, and calculating allergograms were performed as described previously in Chapter 4, Section 4.3.5.

### 5.3.5 Mass spectrometric identification of King prawn TM

A total of 200 µg of soluble protein extract was reduced with dithiothreitol at 60 °C for 30 min, alkylated in the dark with iodoacetamide at 37°C for 30 min. The solution was loaded on a trypsin spin column (Sigma Aldrich, USA), which has been washed, equilibrated and prepared according to the manufacturer's instructions. The samples were incubated for two hours at room temperature and eluted twice with 100 µl of 0.1% formic acid. The eluted solutions were analysed with a UPLC coupled with an ESI interface to a Xevo TQ MS mass spectrometer (Waters Corporation, USA). Digested peptides were separated with a C18 1.7 µm column (Acquity UPLC BEH 300, Waters Corporation) at a flow rate of 0.4ml/min, column temperature 45°C, Gradient 0-1min 2%B; 2.5-35min 10-50%B; 35-47min 50-90%B; 47-52min 90%B; 52-60min 2%B. The composition of solvent A was 0.1% formic acid in water, and solvent B was 0.1% formic acid in acetonitrile sprayer with an applied capillary voltage of 3.0 kV at 120°C. LeuEnk was used as lockspray for mass accuracy reference (lockmass channel). The instrument was operated in low energy MSE (to obtain the precursor ions and their fragmentation data) and data dependent acquisition (DDA) mode over the mass/charge (m/z) range of 50-1950. The spectral acquisition scan rate was 2 seconds with a minimum intensity of 100 or switched off after 3 seconds. Collision energy was ramped in DDA mode for small molecules from 15 to 30 V and for large molecules from 20-40V. The uninterpreted data were processed using Protein Lynx Global Server (PLGS) v2.3 and converted into pkl files. The converted pkl files were searched with mascot database using variable modifications of carbamidomethyl-C N-terminus, deamidation N, deamidation Q and oxidation M. Up to five missed cleavage site were allowed. Search parameters specified were 0.1Da tolerance against the database-generated theoretical peptide ion masses and a minimum of one matched peptide.

### 5.3.6 Basophil activation assay

Basophil activation to prawn allergens was quantified as described previously in chapter 4. The extracts were tested at increasing concentration (0.005 to 5 µg/ml) for activation of basophils in whole blood samples of prawn allergic patients 1 and 9, having low and high shrimp specific IgE values, respectively. Activation of basophils was determined by up-regulation of CD63 surface expression on viable IgE<sup>hi</sup> cells (basophils) by flow cytometry. Positive controls included activation through rabbit anti-human IgE antibody (DAKO Corporation, Carpinteria, CA, USA) to induce IgE cross-linking and f-Met-Leu-Phe (Sigma, St. Louis, MO, USA) to induce IgE-independent activation of basophils. Stimulation buffer alone was used as a negative control. Results were expressed as the percentage of IgE<sup>hi</sup> cells expressing CD63 for each concentration of allergen extract with the negative control level subtracted.

### 5.3.7 Inhibition ELISA

Inhibition assays were performed as described previously in Chapter 4. Sera from patients 1 and 9 were incubated at room temperature for 2 hours with increasing concentrations of raw extract, heated extract (HE1), whole heated extract (HE2) or Bahia grass pollen extract as a negative control. A 96-well polystyrene high binding ELISA plate (Costar, USA) was coated with 1 µg/mL of HE2 extract for 4 hours at room temperature blocked using 5% SMP in PBS-T and exposed to the serum/inhibitor mixture. Bound IgE antibodies were detected using rabbit anti-human IgE polyclonal antibody (DAKO, USA), HRP-labelled goat anti-rabbit IgG polyclonal antibody (Promega, USA) and O-phenylene diamine as substrate. The reaction was stopped using 4M HCl and absorbance measured at 490 nm. Percent inhibition was calculated as  $100 - [(O.D.490 \text{ nm of serum with inhibitor} / O.D.490 \text{ nm of serum without inhibitor}) \times 100]$ .

### 5.3.8 cDNA sequencing of King prawn tropomyosin

cDNA sequencing of tropomyosin (TM) was performed as described in chapter 2. Briefly, total RNA was extracted from king prawns using the RNeasy RNA extraction kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Single stranded cDNA was generated by RT-PCR using a Transcriptor High Fidelity cDNA synthesis kit (Roche, Basel, Switzerland). The generated cDNA was used as a template to amplify the coding region of TM using forward (GCGGATCCGACGCCATCAAGAAGAAGATGC) and reverse (GCGAATTCTTAGTAGCCAGACAGTTCGCTG) primers and cloned into a sequencing vector, pCR 2.1 using the TOPO TA cloning Kit (Invitrogen, Carlsbad, CA, USA).

### 5.3.9 Amino acid sequence alignment of invertebrate tropomyosins

A phylogenetic tree was generated based on the amino acid sequence of invertebrate tropomyosin in order to compare the primary structure variations between the different species. Full length tropomyosin sequences from 60 invertebrate species were obtained from the NCBI database as listed in Appendix Table B 1.5. A multiple sequence alignment of the tropomyosin sequences was performed using Multiple Sequence Comparison by Log Expectation (Muscle). The evolutionary history was inferred using the Neighbour-Joining method.<sup>15</sup> The bootstrap consensus tree inferred from 10000 replicates is taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). The analysis involved 60 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 281 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.<sup>16</sup>

### 5.3.10 Tropomyosin sequence matrix identity comparison

A multiple sequence alignment was performed for invertebrate tropomyosins (Appendix table B 1.5) and the percent sequence identity between each sequence was calculated as compared to the Black tiger prawn sequence using Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). In addition to the full length amino acid sequence comparison, specific IgE epitope regions on prawn tropomyosin previously identified by Reese et al were also selected for epitope specific amino acid sequence alignment.<sup>17</sup> The specific regions selected were Epitope 1 (amino acid residues 43-55), Epitope 2 (amino acid residues 91-101), Epitope 3a (amino acid residues 137-141), Epitope 3b (amino acid residues 144-151), Epitope 4 (amino acid residues 187-197), Epitope 5a (amino acid residues 251-260), Epitope 5b (amino acid residues 266-273) and Epitope 5c (amino acid residues 273-281). The amino acid sequence percent identities were compared for all the selected invertebrate tropomyosins, entire protein and specific epitopes by plotting on an XY scatter plot.

## 5.4 Results

### 5.4.1 SDS-PAGE analysis of the prawn extracts

The different prawn extracts prepared for King prawn and Black tiger prawn were compared using SDS-PAGE (Figure 5.1 A). A prominent band could be observed at 37-39 kDa region for all the prawn extracts, most likely corresponding to tropomyosin. As expected, the raw prawn extracts showed more protein bands as compared to the heated extracts. Interestingly, the two different types of cooked extracts showed different protein profiles in King prawn and Black tiger prawn. The whole heated prawn extracts showed prominent bands in the 70-75 kDa, 37-39 kDa and 15-25 kDa regions, indicating presence of common heat-stable proteins. Several differences could be observed in the banding patterns between the two different prawn species, especially in the 25 kDa region.

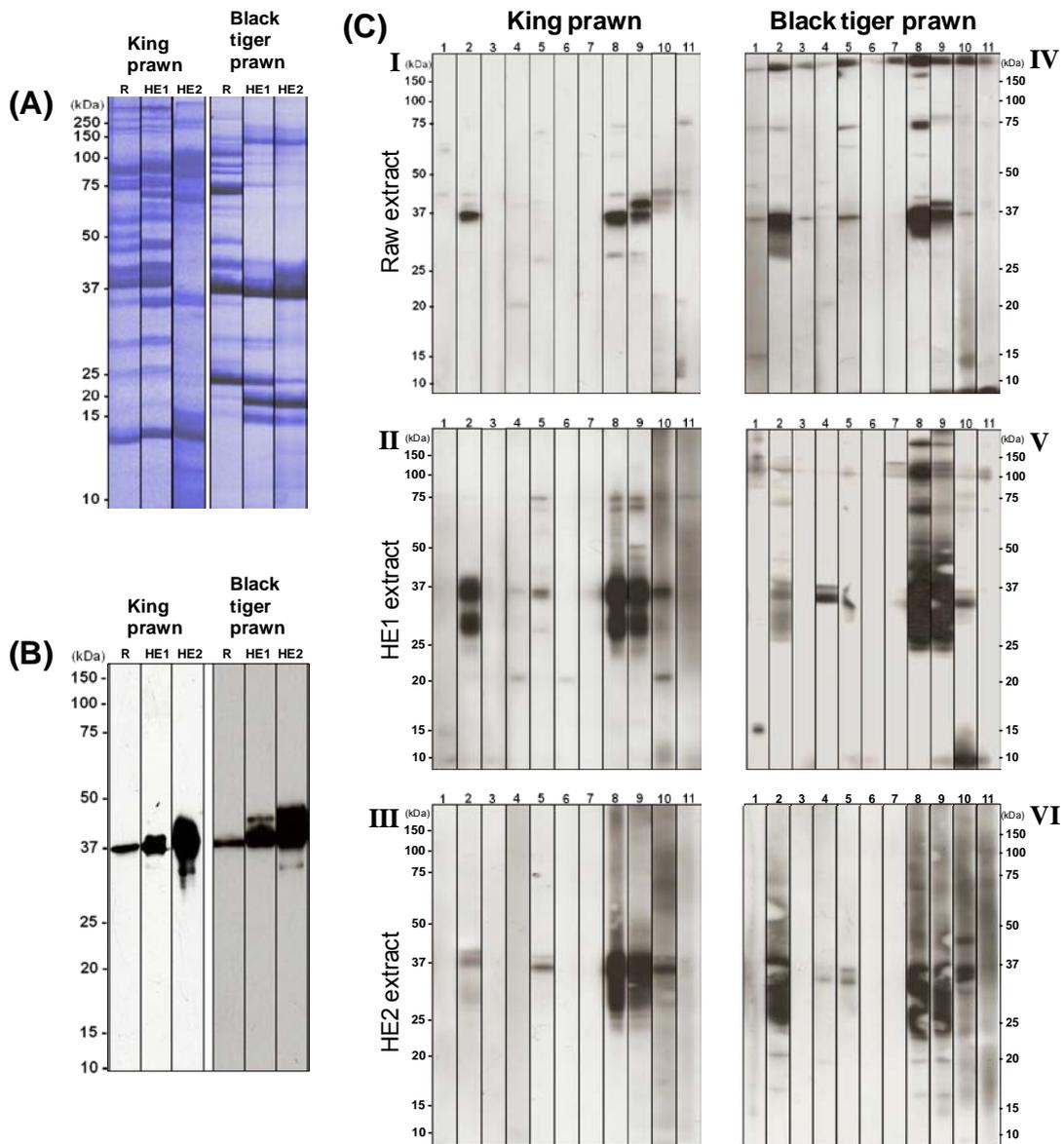
### 5.4.2 Comparison of mAb and IgE antibody binding to three different prawn extracts

Immunoblotting was performed using a monoclonal anti-tropomyosin antibody to visualise the banding pattern in the two prawn species after heat treatment (Figure 5.1 B). In accordance to previously observed data (Chapter 4), tropomyosin was visible as a single band in the raw prawn extract. However after heating, multiple higher bands were observed. Heated extract and whole heated extracts showed a difference in the TM banding pattern, with the latter demonstrating additional higher molecular weight TM band. No differences were observed in the banding pattern between King prawn and Black tiger prawn.

IgE antibody binding to prawn allergens were compared between the two prawn species and its heat treated extracts, using sera from 10 shellfish allergic patients (Figure 5.1 C). Several differences in the IgE binding were observed when compared between King prawn and Black tiger prawn. In the IgE immunoblot to raw extract, a 100 kDa band was observed in Black tiger prawn for most subjects but not in King prawn. IgE binding was observed to a 70 kDa

protein in 50% of the subjects, but stronger for Black tiger prawn. 50% and 70% of subjects tested showed IgE binding to the 37 kDa tropomyosin band in King prawn and Black tiger prawn, respectively. Subjects 1, 3, 5 and 8 showed selective binding to only black tiger prawn tropomyosin in the raw extract. Subject 4 did not elicit IgE binding to TM but to a 20 kDa band which might be myosin light chain or sarcoplasmic calcium binding protein.

Two different types of heat treatment were performed to evaluate the effect of heating on the IgE binding to prawn allergens. An overall increase in the IgE binding intensity was observed to the proteins in the Black tiger prawn and partially in the King prawn heated extracts. A significant difference in IgE binding was observed to the HE1 extracts for the two prawns. IgE binding was observed to two TM bands near the 37 kDa region in the Black tiger prawn extracts in 60% of the subjects. However in King prawns, IgE binding to two distinct bands at 37 kDa and 29 kDa was observed in subjects 2, 8 and 9. Subjects 4, 5 and 10 demonstrated IgE binding to only one band in the 37 kDa region. IgE binding to HE-2 extracts was significantly different compared to HE-1 prawn extracts. In BTP extract, stronger IgE binding was observed to multiple TM bands in subjects who showed binding to TM in the raw extract. Moreover IgE binding to proteins in HE2 extract of King prawns were different to that of Black tiger prawn HE2 extract. In King prawns, IgE binding was observed for two distinct TM bands in the 37 kDa region, however an increase in the binding intensity was not visible.

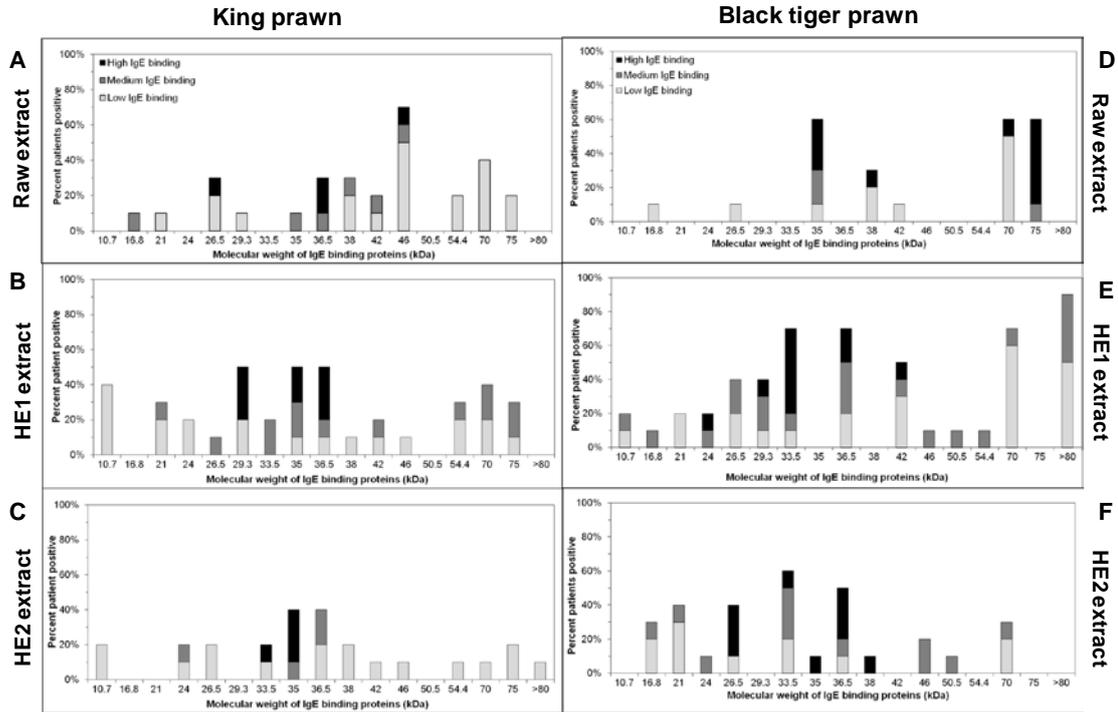


**Figure 5.1:** (A) SDS-PAGE protein analysis and (B) immunoblot using monoclonal anti-tropomyosin antibody for analysis King prawn (*Melicertus latisulcatus*) and Black tiger prawn (*Penaeus monodon*). (C) IgE immunoblot analysis of King prawn (I-III) and black tiger prawn (IV-VI) extracts of 10 shellfish allergic patient sera (lane 1-10) and 1 control non-atopic donor serum (lane 11).

### 5.4.3 Differential IgE binding patterns to prawn extracts and evaluation of heat treatment

Allergogram analysis was performed for the comparative visualisation of the differential IgE binding to King prawn and Black tiger prawn tropomyosin (TM) (Figure 5.2). IgE binding was stronger towards Black tiger prawn TM as compared to King prawn TM as seen at 36.5 kDa in the raw extracts (Figure 5.2 A and 5.2 D). For the HE1 extract, IgE binding could be observed for two distinct bands in the region between 29 kDa and 36.5 kDa in both prawn species. An increase in the IgE binding pattern was observed for lower and higher molecular weight proteins as compared to the raw extracts. A significant difference in the IgE binding pattern was observed between the two types of heated extracts for the two prawns. In HE2, stronger IgE binding was observed to Black tiger prawn TM. IgE binding to King prawn TM was stronger only at 35 kDa. As described previously in Chapter 4, two TM variants were observed after heat treatment in both the prawn extracts. Interestingly, after heat treatment, an increase in IgE binding was observed for BTP TM at 33.5 kDa and 36.5 kDa whereas for KP TM it was observed at 35 kDa and 36.5 kDa.

In addition to tropomyosin, an overall increase in the IgE binding pattern to lower molecular weight proteins was observed. Increased IgE binding was observed in the range of 16 to 26 kDa for the Black tiger prawn HE1 and HE2 extracts. However, no significant increase was observed for King prawn HE1 and HE2.

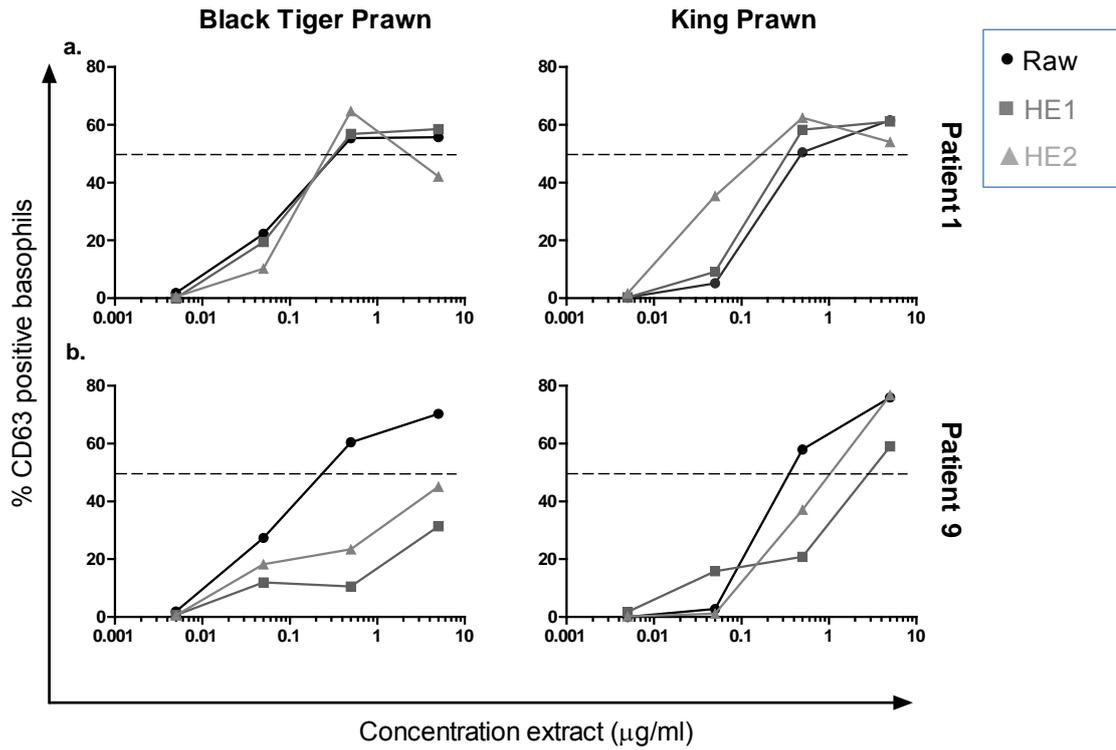


**Figure 5.2:** Allergogram analysis of IgE binding patterns of allergenic proteins in King prawn (*Melicertus latissulcatus*) (A-C) and Black tiger prawn (*Penaeus monodon*) (D-F) extracts. A and D are raw muscle extracts; B and E are heated muscle extracts (HE1); C and F are whole heat treated muscle extracts (HE2).

#### **5.4.4 Evaluation of heat treatment on clinically relevant basophil activation**

Basophil activation test was performed by flow cytometry to assess the biologically relevant IgE reactivity of King prawn and Black tiger prawn extracts. For comparison, two patients were chosen; subject 1 and subject 9 (Appendix table B1.4). Subject 1, having oral allergy syndrome to shellfish, elicited low IgE binding to TM on the immunoblot and had a low sIgE to shrimp on the ImmunoCAP. In contrast, subject 9 was anaphylactic to shellfish and elicited strong IgE binding to prawn TM and high sIgE to shrimp on ImmunoCAP.

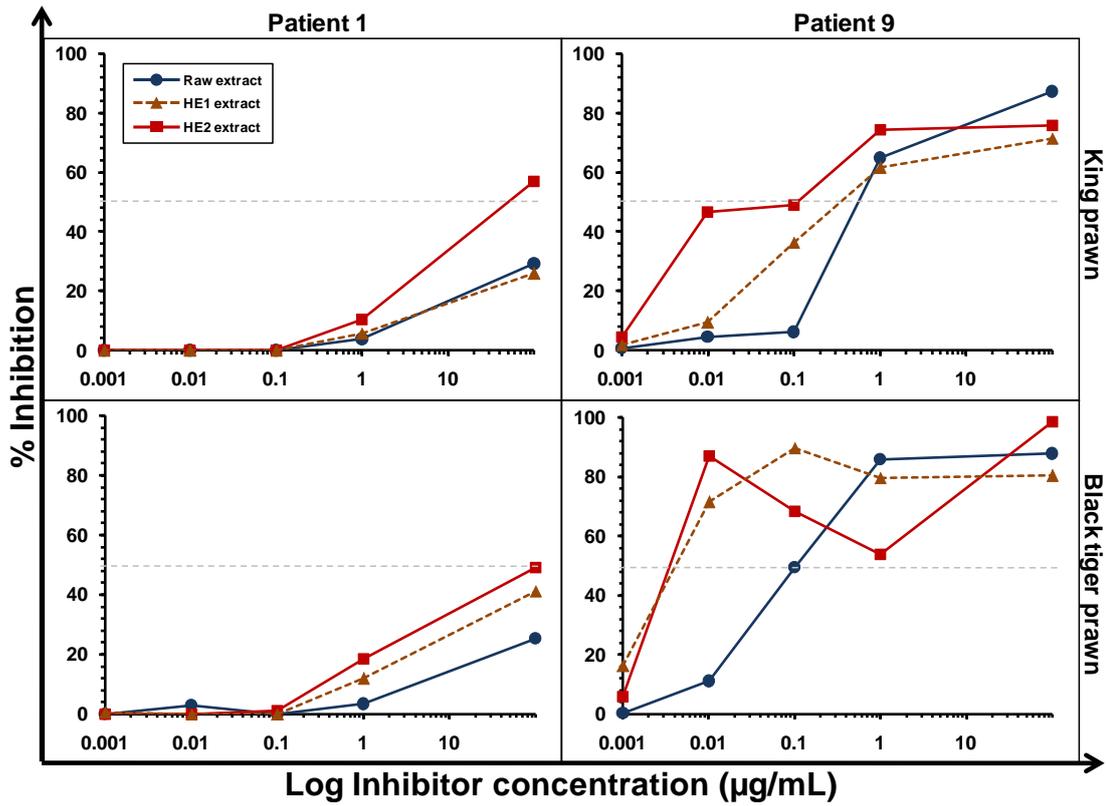
High IgE expression, reactivity and CD63 up-regulation was identified by dose-dependent basophil activation from the subjects' whole blood (Figure 5.3). Significant difference was not observed in the sensitivities of the subjects and basophil activation to the two different prawn species and their respective extracts (Figure 5.3). However, a difference in the sensitivities could be observed when the two subjects were compared to each other for the raw and heated prawn extracts. Subject basophil sensitivities were compared for the different prawn extracts at 50% maximal stimulation. Subject 1 showed higher basophil activation to King prawn heated extract compared to raw extract, but no significant difference in the reactivities to BTP raw and heated extracts. In contrast, subject 9 showed higher basophil activation to both the prawns raw extracts as compared to the heated. Interestingly, the dose dependent activation of basophils was not consistent with the shrimp-specific ImmunoCAP and IgE reactivity on the immunoblot for both the tested subjects.



**Figure 5.3:** Dose response curves for basophil activation of two subjects; 1 and 9 using King prawn and Black tiger prawn extracts. Basophils from whole blood were stimulated with raw prawn (-●-), HE1 extracts (-■-) and HE2 extracts (-▲-) at increasing concentrations. 50% Basophil activation is indicated by a dotted line. Positive controls fMLP (Formyl-Methionyl-Leucyl-Phenylalanine) and anti-IgE were used.

#### **5.4.5 Cross-reactivity of King prawn and Black tiger prawn extracts using Inhibition ELISA**

Inhibition ELISA was performed to evaluate the IgE cross-reactivity among the two prawn species and to understand the effect of heating on IgE cross-reactivity. Black tiger prawn HE2 extract was used as a coating antigen for the inhibition study. IgE inhibition was performed using sera from subject 1 and 9 for comparison purposes. 50% inhibition value of the IgE binding to the coated antigen was used to compare the two prawn extract reactivity (Figure 5.4). No significant difference was observed in the inhibition profile of king prawn and black tiger prawn extract. The heated prawn extracts showed maximum inhibition for both the subjects. However, in case of subject 1 IgE inhibition, a 50% inhibition could not be achieved. This might be attributed to low shrimp sIgE in the subject serum sample. For subject 9 serum IgE, maximum inhibition was obtained using the heated extracts followed by the raw extract. Evidently, the allergen repertoire in the king prawn heated extracts was similar to that of Black tiger prawn. The outcome of the IgE inhibition assay was consistent with the IgE immunoblotting data and shrimp sIgE ImmunoCAP data for subject 1 and 9.

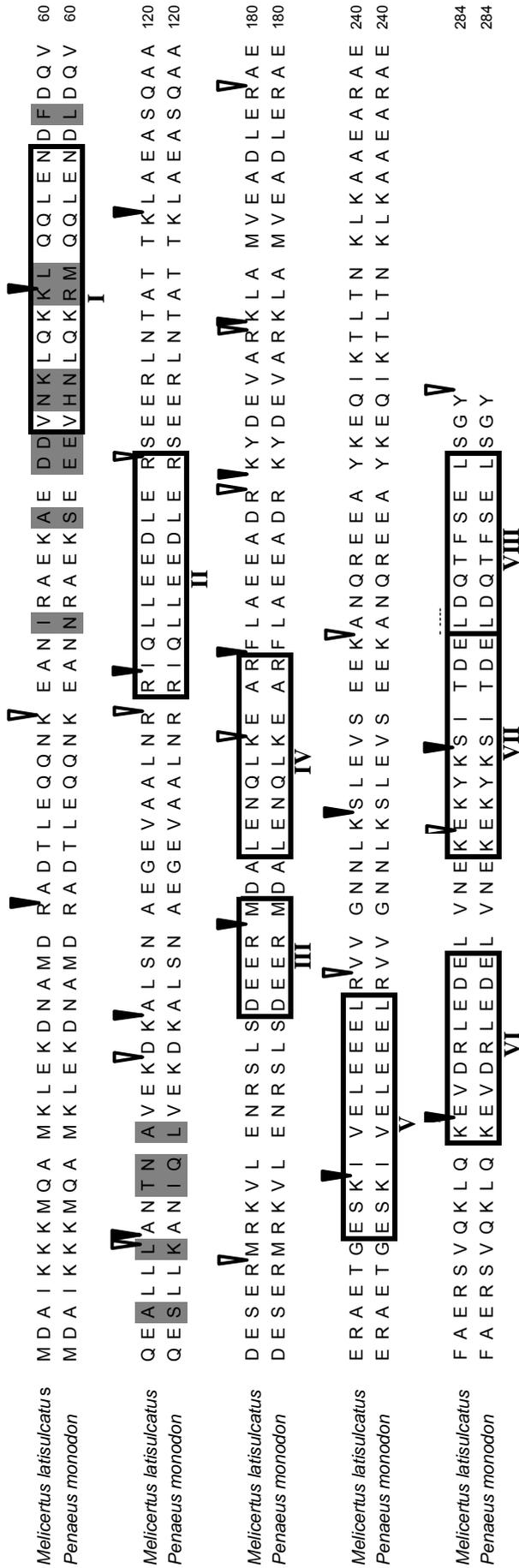


**Figure 5.4:** IgE inhibition ELISA of two subjects 1 and 9. King prawn and Black tiger prawn extracts were used as inhibitors at increasing concentrations; Raw prawn (●), HE1 extracts (▲) and HE2 extracts (■)

#### 5.4.6 King prawn and Black tiger prawn tropomyosin sequence analysis

King prawn and Black tiger prawn TM were cloned and analysed by cDNA sequencing to compare primary structures (Figure 5.5). Fourteen amino acid substitutions were identified in King prawn TM compared to Black tiger prawn TM. Of these 14, six were conserved substitutions and eight were non-conserved substitutions. Interestingly, all the amino acid substitutions were localised near the N-terminal region of tropomyosin, while 4 amino acid substitutions fall within a predicted IgE epitope previously characterised by Ayuso et al 2005.<sup>17</sup>

To confirm the presence of TM in the 36.5 kDa IgE reactive band as shown by immunoblotting, King prawn tropomyosin was identified in the raw and heated extracts using in-gel tryptic digestion and peptide mass fingerprinting (PMF) mass spectrometry. Tropomyosin peptides, identified using this technique, are marked with arrows indicating the N- and C-terminal regions of the peptide in Figure 5.5. The peptide "VNKLQKKLQQLEN" (amino acid residue 43 to 55) was identified which was located in the region with amino acid differences between Black tiger prawn and King prawn. Five other peptides were also detected which fall within the other predicted IgE epitopes. Detection of stable tryptic peptides, originating from highly variable regions of the allergenic protein, may have implications in the design of "signature peptides" as demonstrated in chapter 3.



**Figure 5.5:** Amino acid sequence alignment of King prawn (*Melicertus latissulcatus*), Genbank accession number JX171685 and Black tiger prawn (*Penaeus monodon*), Genbank accession number HM486525.; amino acids marked with grey boxes are variations in the two tropomyosin sequences; black boxes indicate the eight IgE binding regions (I – VIII) previously identified by Reese *et al*; “▼” and “Δ” indicates start and end point of digested peptides identified using mass spectrometry

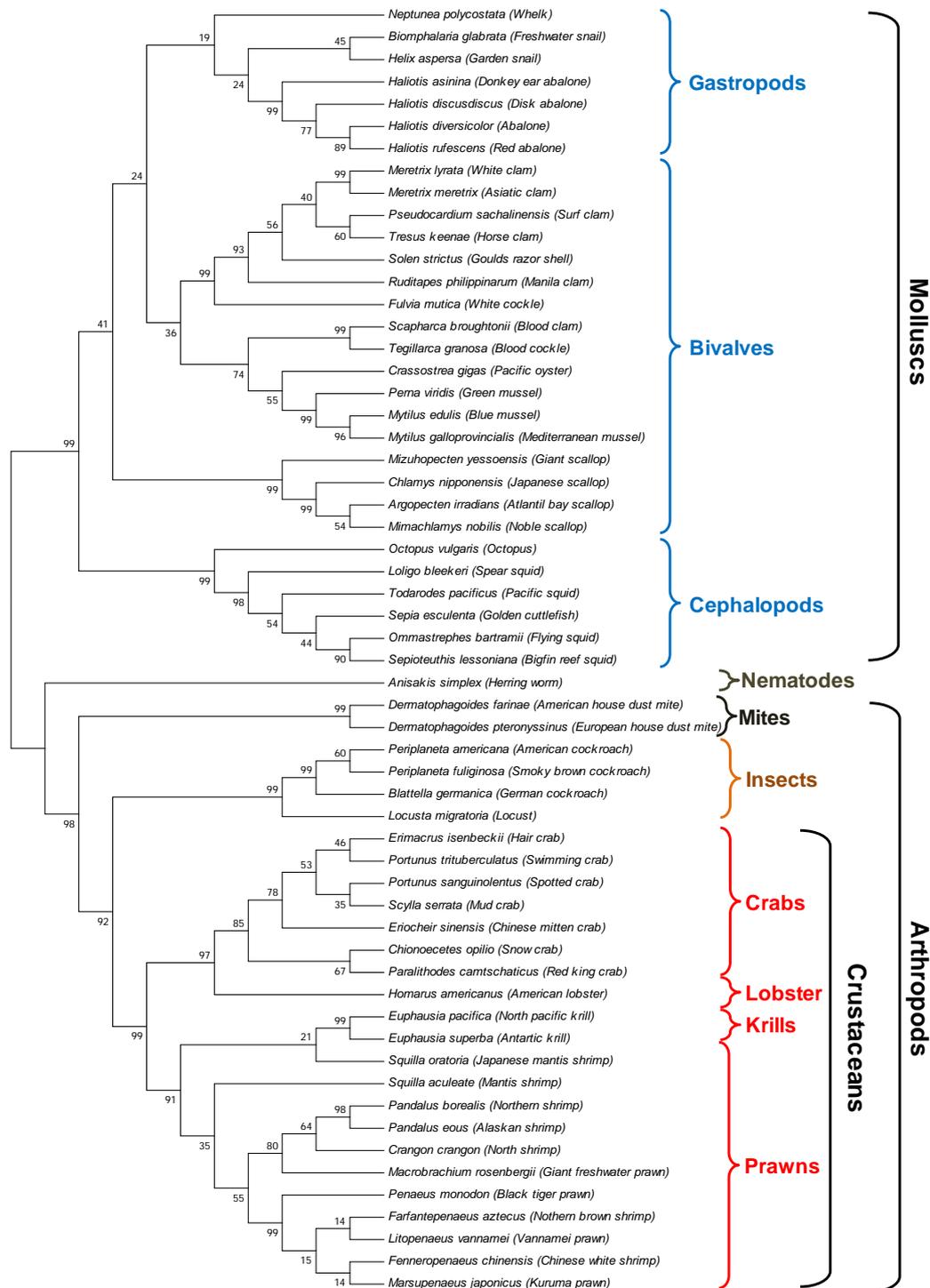
#### 5.4.7 Amino acid sequence comparison of whole tropomyosin and specific IgE epitopes

To corroborate our findings on the comparison of tropomyosin from two different prawn species and to visualise its amino acid sequence identity and conservation, a comprehensive tropomyosin sequence alignment was generated. 60 different tropomyosin amino acid sequences covering the major invertebrate groups were compared as shown in Appendix Table B1.5. A phylogenetic tree based on the relation of tropomyosin primary structure was generated (Figure 5.6). Evidently, a major difference could be seen in tropomyosins from molluscs and arthropods. Interestingly, tropomyosins from bivalves could be divided into two subsections, indicating minor amino acid differences in the bivalve subclasses. Insect TM was closely related to crustacean TM. Among the crustaceans, prawn TM was most closely related to lobster TM. King prawn TM was aligned more towards krill TM as compared to the other crustaceans.

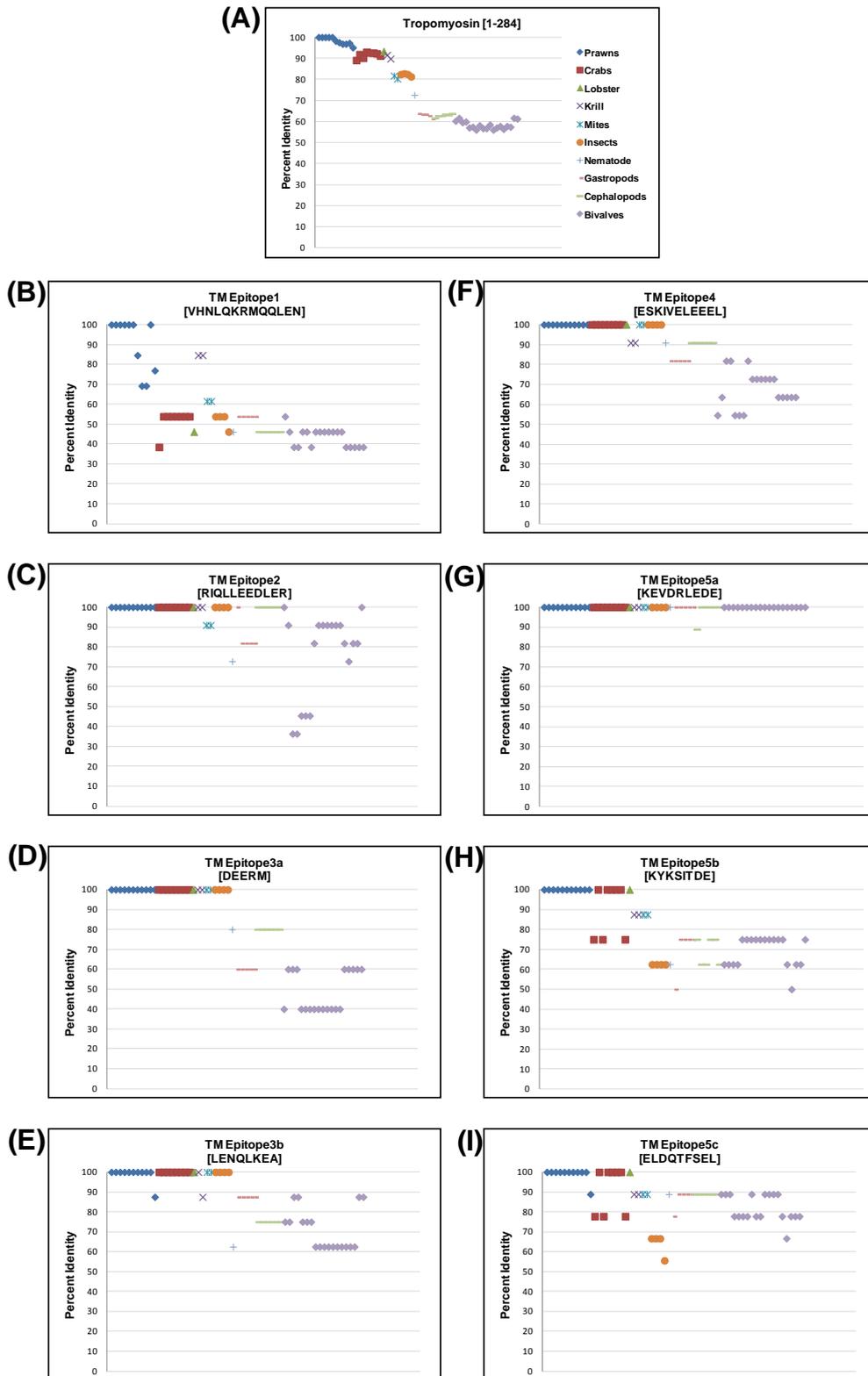
In order to visualise the sequence identity of the specific IgE epitopes, an amino acid alignment was performed and sequence identities were calculated using Clustal O program. Figure 5.7 shows the representation of the sequence identities of eight predicted IgE epitopes using Black tiger prawn tropomyosin as the template against tropomyosins from 60 invertebrate species (Appendix Table B1.5).

When Black tiger prawn TM was compared to other invertebrate TM, the highest sequence identity was observed with crustaceans, in descending order; prawns, crabs, lobster, insects and the lowest to molluscs Figure 5.7A. In IgE epitope 1, the highest variation was observed among the invertebrate TMs with the lowest percent identity of 38% in bivalves (Figure 5.7 B). This was the only IgE epitope of eight where considerable sequence variations were observed among crustaceans, including prawns, crabs, and lobsters, ranging from 40-100%. In epitope 2, a high identity was observed among crustaceans, mites, insects and nematodes and some mollusc species (Figure 5.7 C). However, a wide range of sequence identities was observed among bivalves ranging from 38-100%. A common trend was observed in the sequence variation in epitope

3a, 3b and 4 with most arthropods showing 100% sequence identity and lower but constant sequence identity among the molluscs (Figure 5.7 D, 5.7 E and 5.7 F). Epitope 2, 4 and 5a were observed as the highest conserved IgE epitope out of all predicted IgE epitopes for all the invertebrate tropomyosins (Figure 5.7 G). Except one cephalopod species, all invertebrate species were 100% identical in epitope 5a region of tropomyosin. Epitope 5b and 5c showed sequence variations similar to epitope 1 but with higher sequence identity among crustaceans (Figure 5.7 H and 5.7 I). Interestingly, insect and mite tropomyosin show the lowest sequence identity only in epitope 1, 5b and 5c in comparison to Black tiger prawn TM.



**Figure 5.6:** Phylogenetic tree of tropomyosin from 60 different invertebrate species inferred using Neighbour-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) is shown next to the branches. Clustering of species from the same phyla is shown on the right side of the figures.



**Figure 5.7:** Comparison of amino acid sequence identity of eight IgE binding epitopes. IgE epitope sequences of Black tiger prawn were aligned with tropomyosin sequences of different species and identity is shown as percentage.

## 5.5 Discussion

Clinical cross-reactivity is an important concern related to IgE hypersensitivity. It can lead to clinical reactions not only among different food sources but also to inhalant allergen sources, due to primary sensitisation to a particular food group. Not only does allergen cross-reactivity increase the risk of accidental allergen exposure from unexpected sources, but can also lead to misdiagnosis of allergy in affected patients. Tropomyosin has been shown to be one of the major allergens in invertebrate species and also responsible for clinical and molecular cross-reactivity.<sup>18-20</sup> In this study, the role of tropomyosins primary structure conservation was investigated as a possible explanation for clinical cross-reactivity or non-cross-reactivity among shellfish allergic subjects.

Several studies have attempted to analyse the effect of heating on various food sources.<sup>21-24</sup> Understanding the phenomenon of allergen modification due to heating is important since most food sources undergo some kind of “cooking” before consumption for e.g. roasting of peanuts, cooking eggs, boiling shrimps and lobsters etc. In chapter 4, it was demonstrated how the allergen repertoire of Black tiger prawn is modified due to thermal treatment and its effect on patient IgE binding and reactivity. However, the study was done on a single shellfish species, which may or may not reflect the behaviour of other shellfish allergens.

In this chapter, tropomyosin was the allergen of interest to study the differential patient IgE binding to two prawn species, King prawn (*Melicertus latisulcatus*) and Black tiger prawn (*Penaeus monodon*), a frequently eaten shellfish species. Moreover, the modifications in the IgE binding pattern to prawn extracts with or without heat treatment were analysed and compared for the two prawn species. Finally, a comprehensive analysis and comparison was performed on the tropomyosin IgE epitopes from various invertebrate species to understand the molecular basis for IgE antibody cross-reactivity.

Raw and heated extracts were prepared for both prawn species to evaluate the effects of heating on prawn tropomyosin. A similar increase in binding in tropomyosin bands was observed for both prawn species after heat treatment. Two different methods of heat treatment were employed to test its effects on

subsequent antibody binding patterns. The IgE antibody binding profile to tropomyosin among the heated extracts were different in 40% of the tested subjects for both prawn species. This is of importance when considering that several studies in the past have used different heating methods for shellfish allergen characterisation. It may be possible that use of different types of heated extracts in diagnostic platforms may result in erroneous outcomes and may be non-reproducible. Heat processing of the entire specimen may be the ideal method as it may mimic the natural cooking process. Moreover, most of the shellfish allergen sources used in the ImmunoCAP platform seems to employ this method of heat treatment. However, further testing with patient sera samples from a bigger cohort would be necessary to validate the best type of heating method to be used for allergy diagnostics.

The IgE antibody binding pattern to tropomyosin was analysed using sera from 10 shellfish allergic subjects. A significant difference in the IgE binding pattern was observed between the two prawns for raw and heated extracts. However, the difference was not a positive or negative IgE binding signal among patients to the different prawn species but rather a difference in the IgE antibody binding intensities to single or multiple isoforms of the allergenic proteins. Similar to the observation in Chapter 4, there was a significant increase in the overall IgE binding after heat treatment to both prawns. Interestingly, the whole heated king prawn extract did not show a significant increase in IgE binding as compared to the Black tiger prawn.

Using allergogram analysis and mass spectrometric identification, IgE binding to tropomyosin was observed to both prawn species. Similar to Black tiger prawn, an increase in IgE binding to two tropomyosin variants was observed in the heated extracts. However, the percent subjects binding to the King prawn tropomyosin variants (4/10) differed from those binding to Black tiger prawn tropomyosin (7/10). This suggested different recognition of the two tropomyosins by the subject's IgE antibodies.

Basophil activation studies were performed to further investigate the differential IgE binding to King prawn and Black tiger prawn tropomyosin and its biological relevance. Interestingly, the subject with high sIgE to tropomyosin showed least

basophil reactivity to the heated extracts while the subject with low sIgE to tropomyosin showed higher basophil sensitivity to heated as compared to raw extract. This observation was not expected since heated extracts contains multiple IgE binding tropomyosin variants compared to the raw extract as confirmed by IgE immunoblotting. This data may point to the fact that some patients may have IgE antibodies which are targeted to specific regions of tropomyosin with varying avidity and affinity to the IgE epitopes and which in turn would lead to either strong or weak recognition, with subsequent reactivity. In addition, since whole extract preparation was used in the basophil activation studies, other prawn allergens may have played a role in sensitisation of the basophils. Future analysis with either natural purified or recombinant tropomyosin will corroborate the current findings.

The ELISA inhibition assays confirmed the King prawn heated extracts to have a similar allergen repertoire as compared to Black tiger prawn extract. Importantly, higher inhibition was observed using Black tiger heated extracts compared to King prawn heated extract.

To further investigate the role of primary structure of the allergen, King prawn tropomyosin was sequenced and compared to Black tiger prawn tropomyosin. Interestingly, the two tropomyosins were found to be 95% identical. In contrast, previously characterised prawn tropomyosins share a 99-100% identity. This difference of King prawns may be because of two reasons; firstly, a different isoform of tropomyosin may be expressed more abundantly in King prawn as compared to other species, or secondly, King prawn may be evolutionarily apart from the genus, *Penaeus*. It is noteworthy that King prawn tropomyosin was found to be more similar to lobster tropomyosin. Most of the amino acid substitutions between the two tropomyosins were located near the N-terminal region. Interestingly, the amino acid substitutions appear to be located in an IgE epitope previously characterised by Reese et al.<sup>14</sup> In this region, four amino acid substitutions were found in King prawn tropomyosin when compared to the Black tiger prawn tropomyosin, which included two non-conservative substitutions. Reese et al have demonstrated that at least three amino acid substitutions were necessary to abolish or decrease IgE binding activity. Moreover, the IgE epitope "VHNLQKRMQQLLEN" has been shown to be one of

the major IgE epitopes with four test subjects eliciting IgE binding.<sup>17</sup> It can therefore be concluded that substitutions in this region of tropomyosin may be responsible for the differential IgE binding to King prawn as compared to Black tiger prawn among the 10 tested subjects.

A preliminary amino acid sequence alignment analysis in chapter 2 had revealed that most of the substitutions are located in the N-terminal region of tropomyosin. This could now be confirmed in the sequence comparison of King prawn and Black tiger prawn tropomyosin. Several studies on cross-reactivity of tropomyosin from various species have often looked at the entire protein sequence. Due consideration was not given to the sequence variation in the specific IgE epitopes of tropomyosin. In this study a sequence alignment of a comprehensive panel of known and sequenced set of invertebrate tropomyosins was performed and sequence identities compared for the IgE epitope regions. Interestingly, a wide range of conservation or variation could be seen among the different IgE epitopes. Clearly, some epitopes reflected very high variability especially in crustaceans. In contrast, IgE epitopes 2, 4 and 5a showed high amino acid conservation across arthropods and molluscs. Four out of eight epitopes showed a difference in sequence identities among arthropods and molluscs; however a strong conservation was observed within the specific groups. These similarities in the IgE epitopes of tropomyosin among the various invertebrate species may explain the observed mono-sensitisation or multiple-sensitisation to food and inhalant allergens from different invertebrate sources, such as dust mites and insects.

Previous studies on tropomyosin B-cell epitopes have concluded that the motif LEXXL, where L is leucine and X is a negatively charged amino acid, to be the allergenic motif.<sup>14, 17</sup> However, it is not yet known why or how different patients have a different repertoire of specific IgE antibodies against multiple IgE epitopes. The current study highlights the variability in the primary structure of different tropomyosins in specific IgE epitopes and its possible effects on IgE binding and biological reactivity in allergic patients.

Several studies have been conducted in the past to evaluate the significance and use of tropomyosin as a marker of allergy and severity of symptoms.<sup>13, 25-27</sup>

It has been shown that more than 60% of shellfish allergic patients demonstrated IgE reactivity to tropomyosin. However, in this study IgE binding to TM could not be correlated to the severity of symptoms as shown in chapter 4. Moreover, the use of entire allergenic proteins or grafted IgE epitopes rather than simple peptides has shown to be efficient at IgE binding.<sup>25</sup> The findings of the current study might assist towards the design and use of specific allergens for component resolved diagnostics.

In summary, the differential IgE binding to tropomyosin from King prawn and Black tiger prawn was analysed. IgE binding was different to tropomyosin from the two prawn species. Heating had an effect on IgE binding to King prawn tropomyosin but different to Black tiger prawn tropomyosin. King prawn tropomyosin was found to be 95% identical to Black tiger prawn tropomyosin, with four amino acid substitutions covering one major IgE binding epitope. This modified region may have been responsible for the differential IgE reactivity to tropomyosin from the two prawn species. Finally, a comprehensive amino acid sequence comparison was performed to evaluate the variation or conservation of the IgE binding epitopes of invertebrate tropomyosins.

In the next chapter, tropomyosin is identified and characterised from a frequently consumed crab species in the Asia-Pacific region using a larger patient cohort. Furthermore, the novel crab tropomyosin IgE cross-reactivity to Black tiger prawn tropomyosin is evaluated. The biological relevance of IgE binding to tropomyosin is evaluated using basophil activation assays using recombinant allergens.

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## 5.7 Chapter 5 summary

- Shellfish tropomyosin elicits immunological cross-reactivity among allergic patients. The aim of this chapter was to investigate differential IgE binding to tropomyosin from raw and heated extracts of two different prawn species, and to investigate the molecular basis of IgE cross-reactivity by analysing an extensive epitope-specific multiple sequence alignment of different tropomyosins
- IgE binding to tropomyosin was considerably different to tropomyosin between the two prawn species
- Heating had an enhancing effect on IgE binding to King prawn tropomyosin but the repeated pattern was different as compared to Black tiger prawn tropomyosin
- King prawn tropomyosin is 95% identical to Black tiger prawn tropomyosin, which four amino acid substitutions in one major IgE binding epitope
- Using multiple sequence alignments, IgE binding epitope regions 2 (amino acid residue 91-101), 4 (187-197) and 5a (251-259) were identified to be highly conserved among crustacean and mollusc species and may be responsible for observed immunological and clinical cross-reactivity.

## CHAPTER 6

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### IGE REACTIVITY OF BLUE SWIMMER CRAB (*PORTUNUS PELAGICUS*) TROPOMYOSIN, *POR p 1*, AND OTHER ALLERGENS; CROSS-REACTIVITY WITH BLACK TIGER PRAWN AND EFFECTS OF HEATING

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## 6.1 Introduction

In the previous chapters, tropomyosin has been confirmed as the major heat-stable crustacean allergen.<sup>1-4</sup> Other allergens derived from muscle tissue were identified, including myosin light-chain, arginine kinase and sarcoplasmic Ca-phosphate isomerase.<sup>4-8</sup> However, only a few species have been studied so far, mostly prawns, with few reports on crab allergens.<sup>9</sup> Only one crab allergen, TM from the crucifix crab (*Charybdis feriatus*), is published in the IUIS allergen database (<http://www.allergen.org/index.php>). Furthermore, there is little information on shellfish from the southern hemisphere or Asia-Pacific region. Patients frequently report clinical reactions to more than one shellfish species, but whether this is a result of multiple sensitivities or from IgE cross-reactivity between allergens of different shellfish species is unknown.<sup>10, 11</sup> This information is vital for optimal management of shellfish allergy.

Adding complexity, there are reports of altered stability and allergenicity of food proteins after processing.<sup>12-14</sup> As shown in Chapter 2, Chapter 4 and other studies, most members of the TM allergen family are highly heat-stable.<sup>3, 8, 15, 16</sup> In Chapter 4, the effect of heating on allergens in whole prawn extract was analysed. However, there is still a lack of information on the effects of heating on whole shellfish extracts<sup>17</sup>, with most studies testing heated purified allergens. As described earlier, heating can enhance allergenicity through several mechanisms including protein denaturation and exposure of new epitopes, aggregation and chemical modification such as advanced glycation end products through the Maillard reaction.<sup>8, 18</sup>

The current chapter reports the characterisation of allergenicity of a commonly consumed crustacean species, the blue swimmer crab (*Portunus pelagicus*), and in particular the identification of the major allergen *Por p 1*. Evidence of cross-species IgE reactivity with another commonly consumed species, the Black tiger prawn (*Penaeus monodon*), was sought and the effect of heating on allergens of both species and their cross-reactivity was assessed. Clinically relevant IgE reactivity to the shellfish extracts was assessed using whole blood basophil activation assay.

## 6.2 Aims

The specific aims for the work described in this chapter were

- To identify and characterise the major allergen tropomyosin in a commonly consumed crab species, the Blueswimmer crab *Portunus pelagicus*.
- To investigate the impact of thermal treatment on the IgE reactivity of crab tropomyosin.
- To evaluate the IgE antibody cross-reactivity of Blueswimmer crab tropomyosin with a well characterised Black tiger prawn tropomyosin
- To evaluate the clinical relevance of the IgE cross-reactivity of crab and prawn extracts using the basophil activation assay.

## 6.3 Materials and Methods

### 6.3.1 Ethics statement

Informed written consent was obtained from all subjects, with ethics approvals from the Alfred Hospital Research Ethics Committee (Project number 192/07) and the Monash University Human Ethics Committee (MUHREC CF08/0225).

### 6.3.2 Study Population and Sera

Serum samples were obtained from twenty-four shellfish-allergic subjects (mean age  $32 \pm 10.5$  years; 13/24 female), seven non-atopic controls (mean age  $40.3 \pm 12.3$  years; 4/7 female) and one atopic non-shellfish-allergic subject (age 28 years, female). Allergic subjects were identified from the Alfred Hospital Allergy clinic seafood allergy database on the basis of clinical history of allergy to shellfish and positive shrimp-specific IgE (ImmunoCAP [Phadia Pty Ltd, Uppsala, Sweden]  $>0.35\text{kUA/L}$ ) (Table 6.1). Of these subjects, 18/24 (75%) were also positive for crab-specific IgE. Eight control subjects were selected on the basis of no clinical history of shellfish allergy; seven were non-atopic, i.e. had a negative skin prick test response to a basic panel of aeroallergens and one was atopic (Bahia grass pollen-sensitised).

### 6.3.3 Preparation of Shellfish Extracts

Fresh Blue swimmer crab (*Portunus pelagicus*) and Black tiger prawn (*Penaeus monodon*) were purchased at the Prahran market (Melbourne, Australia). Extraction was performed as described in Chapter 2. For raw crab (RC) and raw prawn (RP) extracts, the outer shell was removed and muscle collected. Finely cut muscle was blended with PBS pH 7.2 and left overnight at  $4^\circ\text{C}$  with constant mixing. The crude extract was centrifuged at  $13,000\text{ g}$  at  $4^\circ\text{C}$  for 20 min. Supernatant was collected and filter sterilised before storage at  $-80^\circ\text{C}$  in aliquots. For heated crab (HC) and heated prawn (HP) extracts, the outer shell was kept during the heating process (20 min immersed in boiling PBS) before removal and extract preparation as above. The protein concentration of each

extract was determined using the Bradford assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as a standard.

#### **6.3.4 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

SDS-PAGE was performed as previously described in chapter 2 with the following modifications. Proteins of shellfish extracts, 15 µg/lane, were separated under reducing conditions using 4-12% Bis-Tris gels (NuPage, Carlsbad, CA). Pre-stained standards (1x See Blue Plus2, Invitrogen, Carlsbad, CA) were used as molecular weight markers. Proteins were resolved at 200 V for 35 min using an Xcell II mini-cell apparatus (Invitrogen) and the gel was stained with Coomassie brilliant blue.

#### **6.3.5 IgE ELISA and Inhibition IgE ELISA**

Wells of a 96-well EIA/RIA plate (Costar, St. Louis, MO) were coated with 100 µL extract (RC, HC, RP or HP; 1 µg/mL in PBS, or PBS alone for no-antigen control wells), and incubated overnight at 4°C. All of the following incubations were performed for 1 h unless otherwise stated and the plate was washed 4 times in 0.05% Tween 20/PBS (PBS-T) between steps. Blocking was performed using 5% skim milk diluted in PBS-T. Serum, 100 µL diluted 1:10 in 1% skim milk/PBS-T, was added to wells and then incubated for 3 h at room temperature with shaking (45 rpm). Rabbit anti-human IgE antibody (1:4000; Dako, Glostrup, Denmark) and goat anti-rabbit IgG-HRP (1:1000; Promega, Madison, WI) were added sequentially to wells and plates incubated at room temperature for 1 h with gentle shaking. Plates were then washed 5 times in PBS-T, followed by 3 washes in PBS. IgE binding was detected using TMB (3,3',5,5'-Tetramethylbenzidine) substrate (Invitrogen). After 5 min, the reaction was terminated using 1M HCl and the absorbance (O.D.) at 450 nm measured by spectrophotometry (Thermo Fisher Scientific, Melbourne, Australia). Seven non-atopic subjects were screened to determine the extent of non-specific binding. No-antigen, background values were subtracted from test sera data.

The cut-off for positive IgE binding in shellfish-allergic subjects was two standard deviations above the mean O.D.450nm of the non-atopic subjects.

For inhibition ELISA experiments, individual subject sera were first titrated (1:10-1:1280) for IgE reactivity with HC or HP to determine the concentration at which the O.D.450nm was ~1 and within the linear phase of the titration curve. Using this dilution, serum samples were incubated with an equal volume of shellfish extract (RC, HC, RP or HP at 0.16, 0.8, 4, 20 and 100 µg/mL) or purified recombinant TM from black tiger prawn (*rPen m 1.0101*) (at 0.048, 0.24, 1.2, 6, and 30 µg/mL) for 1 h at room temperature and then tested for reactivity with either HC or HP extracts. Percent inhibition was calculated using the following equation: percent inhibition =  $100 - [(O.D.450 \text{ nm of serum with inhibitor} / O.D.450 \text{ nm of serum without inhibitor}) \times 100]$ . For comparison of inhibition by different extracts, the extract (inhibitor) concentration that caused 50% inhibition was calculated from dose-response curves. To assess non-specific inhibition by extracts, serum from a non-shellfish-allergic atopic (Bahia grass pollen-sensitised) subject was incubated with the above inhibitors, and then tested for IgE reactivity with Bahia grass pollen extract as an inhibitor in comparison with untreated serum.

### 6.3.6 IgE Immunoblot

IgE Immunoblotting was performed as described previously in chapter 4 with the following modifications. Proteins of each of the four extracts (RC, HC, RP, HP) were separated by SDS-PAGE as above with 3 µg/well loaded into a 10 or 15-well 4-12% Bis-Tris gel (NuPage), or 60 µg protein loaded into a 6 cm 2D well 4-12% Bis-Tris gel (NuPage). Proteins were transferred to a nitrocellulose membrane (0.45 µm; Thermo Scientific, Rockford IL) using an Xcell II blotting apparatus (Invitrogen) at a constant voltage of 30 V for 1 h. Successful transfer of protein was evaluated by Coomassie brilliant blue staining of the gel as above. The membrane was blocked using 5% skim milk/PBS-T for 1 h at room temperature with gentle rocking. Subject serum (1:40 in 5% SMP/PBS-T) was then applied to the membrane using a Miniblotter apparatus (Immunelect, Boston, MA). To detect IgE binding, the immunoblot was incubated sequentially

with rabbit anti-human IgE antibody (Dako; 1:15,000) and goat anti-rabbit IgG-HRP conjugated antibody (Promega; 1:15,000) each for 1 h at room temperature with gentle horizontal shaking. Following incubation with chemiluminescent peroxidase substrate (Sigma-Aldrich, St. Louis, MO) proteins were visualised using enhanced chemiluminescent technique.<sup>19</sup> TM bands were identified in parallel immunoblots using a rat anti-TM monoclonal antibody (mAb; 1:6000; Abcam, Cambridge, UK) followed by rabbit anti-mouse IgG-peroxidase antibody (1:80,000; Sigma-Aldrich) and development as above.

### 6.3.7 Sequence analysis of Blue Swimmer Crab Tropomyosin

A band corresponding to the predominant IgE-reactive 39 kDa protein was excised from the SDS gel for mass spectrometric analysis. The band was de-stained, reduced, alkylated and digested with trypsin as reported previously.<sup>4</sup> Digested protein was injected into a DIONEX Ultimate 3000 liquid chromatography system (Germering, Germany) coupled with a QSTAR XL hybrid quadrupole-quadrupole/time-of-flight tandem mass spectrometer (QqToF-MS/MS; Applied Biosystems/MDS Sciex, Foster City, USA). The resultant tandem spectra were searched using the Matrix Science (Mascot) search engine (precursor and product ion mass tolerance set at 0.1 Da). For cloning and full sequencing of the crab TM, total RNA was extracted from crab pincer muscles using Trizol reagent (Invitrogen) following the manufacturer's instructions. Single stranded cDNA was reverse transcribed from the RNA using RT-PCR with a cDNA synthesis kit (Bioline, Sydney, Australia). Oligo(dT) primers were used for the reverse transcription. Due to the high amino acid sequence identity of the N and C-terminal regions of tropomyosin among crustacean species, crab tropomyosin specific primers were designed based on the amino acid sequence of the homologous tropomyosin sequence from Rock lobster, previously characterised by our group; (Rock lobster *Jasus lalandii* accession number JX860677.1). The TM specific cDNA region was amplified using PCR with the primer pair BSC-TM (F) 5'-GHCGGATHC-ATGGACGCAATCAAGAAGAAGATGCAG-3' and BSC-TM (R) 5'-GCGGAATTC-TTAGTAGHCAGACAGTTCG-3'. The PCR included one cycle of

denaturation at 94°C for 2 minutes, 35 cycles at 94°C for 30 seconds, annealing at 55°C for 45 seconds and elongation at 72°C for 1 minute, and a final elongation step at 72°C for 7 minutes. The amplified full length open reading frame was cloned into the sequencing vector, pCR 2.1 (Invitrogen) using the *BamH1* and *EcoR1* restriction sites for cloning, and the open reading frame for TM sequenced (Macrogen Inc, Seoul, South Korea) to obtain the construct pCR2.1\_TM.

### 6.3.8 Expression and purification of recombinant crab tropomyosin

Expression and purification of recombinant crab tropomyosin was performed as described previously in Chapter 2. Briefly, the open reading frame of tropomyosin was cross-cloned from the construct, pCR2.1\_TM to the expression vector, pProEXHT-B using restriction sites for *BamH1* and *EcoR1* and ligation was performed using T4 DNA Ligase (Invitrogen, CA, USA). This expression plasmid construct was transformed into chemical-competent NM522 *E.coli* cells using heat-shock for 30 seconds, incubation in SOC medium at 37°C for 1 hour and grown overnight on Luria Bertani (LB) agar plates with 100 µg/mL ampicillin (Amresco, USA) at 37°C. Positive colonies were tested using PCR as described above and selected for protein expression.

For recombinant protein expression, 5 mL of fresh overnight culture from a single colony was used to initiate growth in 1 L LB broth containing 100 µg/mL ampicillin. Recombinant protein expression was induced using 0.6 mM isopropylthio-β-galactoside, IPTG (Amresco, USA). After expression, the culture was centrifuged at 3500 g for 10 mins to obtain the bacterial pellet and subsequently resuspended in extraction buffer (25 mM Tris-HCl, 300 mM NaCl, 1 mM imidazole, pH 8). Recombinant crab tropomyosin containing the 6xHis tag was extracted from the *E.coli* cells using a French-Pressure Cell and purified using nickel charged metal-chelate affinity chromatography (GE Healthcare, USA) following the manufacturer's instructions and stored at -80°C until further use.

### **6.3.9 Whole Blood Basophil Activation Test**

The basophil activation test was performed as described in Chapter 4. Briefly, shellfish extracts were tested at 0.01-10 µg/mL for activation of basophils from heparinized whole blood samples of five shellfish-allergic subjects and one non-atopic control subject. This was based on analysis of up-regulation of the cell marker CD63 on IgE-positive basophils by flow cytometry as described previously.<sup>20</sup>

### **6.3.10 Statistical analysis**

The Wilcoxon matched-pairs signed rank test was used to compare overall serum IgE reactivity between shellfish extracts, and Spearman's correlation test was used to assess correlation between individual specific IgE levels against different extracts or using different assays. Analyses were performed using GraphPad Prism version 5.04 for Windows (GraphPad, San Diego, CA).

**Table 6.1:** Clinical features of subjects with allergy to shellfish

Subject ID	Age (yrs)	Sex	Total IgE (IU/mL)	Crab specific IgE (kU <sub>A</sub> /L)	Shrimp specific IgE (kU <sub>A</sub> /L)	Clinical presentation to shellfish Symptoms	Known shellfish species
1	19	F	242	1.17	1.32	As, R, U	Prawn
2	45	M	136	2.77	4.54	O	Prawn
3	25	M	158	0.57	0.85	R, U, An	Prawn
4	28	F	238	6.05	5.93	A, An	Prawn
5	23	M	283	18.1	19.7	As, R, O, U	Prawn
6	22	F	3401	3.36	6.65	As, R, U, An	Prawn
7	47	M	822	2.72	5.37	A, U	Prawn and all crustaceans
8	24	F	1946	9.5	2.42	R, A, O	Prawn, crab
9	35	F	3887	2.94	3.33	O, U	Shrimp
10	39	M	192	0.06	1.22	R, O	Raw prawn
11	44	M	976	8.21	9.03	R, O	Squid
12	23	M	658	4.56	5.14	R, U, An	Prawn
13	26	M	127	0.15	1.65	O	Prawn
14	55	F	566	0.13	1.36	As, R, O	Raw prawn, crab Prawn, lobster, crayfish
15	37	M	194	0.2	1.41	R, O	Prawn, lobster
16	47	F	92	0.01	1.41	R, O	Shellfish
17	32	F	28	8.42	9.82	O, U	Crustaceans
18	30	F	748	0.63	2.84	R, U	Crustaceans
19	32	M	183	3.09	6.84	An, O	Prawn
20	22	F	164	0.01	1.43	As, R	Prawn
21	17	F	1550	58.3	60.8	U, An As, R, U,	Prawn
22	22	M	81	2.37	2.57	An, A	Crustaceans
23	44	F	167	22.9	32.4	As, R, U	Prawn
24	31	F	130	6.97	8.98	O	Crab

F: female, M: male. NT: not tested.

As: asthma, R: rhinitis, A: anaphylaxis, U: urticaria, An: angioedema, O: oral/facial symptoms

## 6.4 Results

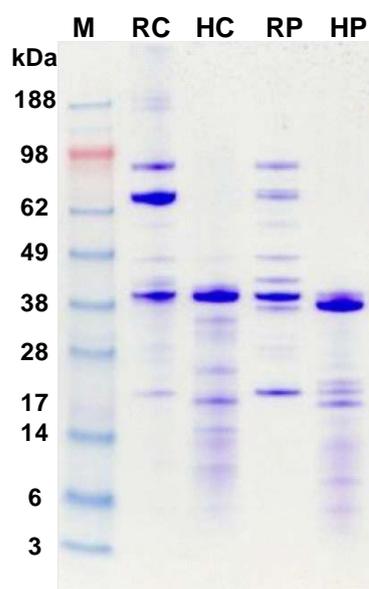
### 6.4.1 SDS-PAGE Analysis of Shellfish Extracts

Analysis of raw and heated shellfish extracts by SDS-PAGE and Coomassie brilliant blue staining (Figure 6.1) revealed an array of proteins ranging from about 6 to 188 kDa. A prominent protein band at 37-39 kDa was seen in all extracts, consistent with TM (34-39 kDa). Other bands corresponded to the known shellfish allergens arginine kinase (about 42 kDa), myosin light chain, sarcoplasmic calcium binding protein and troponin C (about 21 kDa). However several other bands were also apparent which do not correspond to known shellfish allergens. Some differences could be seen between the RC and RP extracts, most notably the strong band at 69 kDa seen strongly in the RC but only weakly in the RP. In addition, there was only one major protein band in the TM region in RC, whilst there were two bands in RP. More pronounced differences were seen when raw and heated extracts of both species are compared. For both HC and HP extracts, the higher molecular weight proteins seen in the raw extracts were not present, most likely due to protein degradation during the heating process. This is supported by the appearance of lower (<35 kDa) molecular weight proteins only present in the heated extracts. The actual sizes of these lower proteins differed between the crab and prawn extracts. The molecular weight of the prominent TM region band for the prawn extract decreased from 39 kDa to 37 kDa on cooking, but did not change for the heated crab extract, remaining at 39 kDa.

### 6.4.2 ELISA for serum IgE reactivity to shellfish extracts

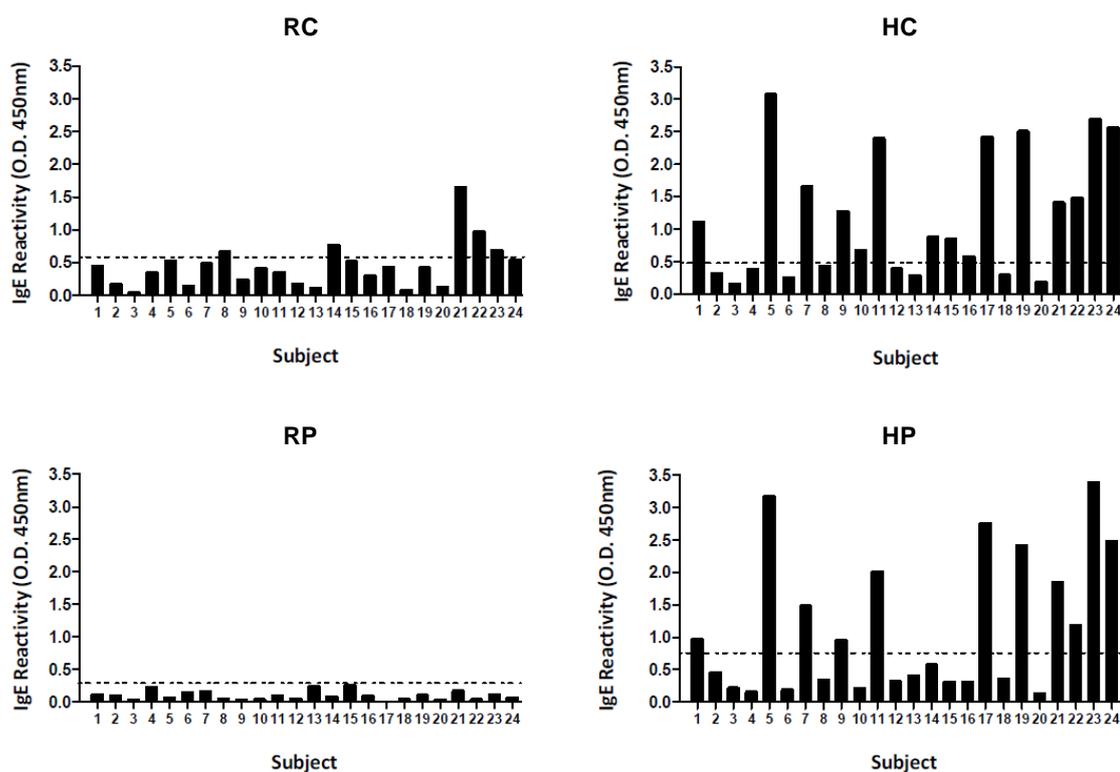
Quantitation of serum IgE binding to the shellfish extracts by ELISA (Figure 6.2) showed that the heated extracts have markedly higher IgE reactivity than the corresponding raw extracts. Median O.D. values for HC and RC were 0.86 and 0.41, respectively (HC vs RC  $p < 0.001$ ) and for HP and RP were 0.51 and 0.08, respectively (HP vs RP  $p < 0.001$ ). The RC extract was significantly more IgE reactive than RP ( $p < 0.001$ ), but there was no overall difference between the two heated extracts. Of the 24 shellfish-allergic subjects, 5 (21%) had

positive IgE reactivity to RC, 15 (63%) to HC (including 4 of the 5 RC positives), none to



**Figure 6.1:** SDS-PAGE analysis of shellfish extracts. 4-12% SDS-PAGE of whole shellfish extracts stained with Coomassie brilliant blue. M, molecular weight markers; RC, raw blue swimmer crab; HC, heated blue swimmer crab; RP, raw black tiger prawn; HP, heated black tiger prawn

RP, and 11 (46%) to HP. A similar pattern of reactivity was observed between the HC and HP extracts. All subjects who were positive to HP were also positive to HC, and of those positive to HC but not to HP, reactivity was only weak (10, 14, 15 and 16). These same four subjects had a negative crab ImmunoCAP. Overall there was a significant correlation between IgE levels by ELISA for the HC and HP and the relevant ImmunoCAP values ( $p < 0.01$ ), but not for the raw extracts. However, several subjects showed a lack of concordance of positive or negative result for ELISA with heated extracts and ImmunoCAP.



**Figure 6.2:** ELISA for serum IgE reactivity to shellfish extracts. ELISA for serum IgE reactivity to raw blue swimmer crab (RC), heated blue swimmer crab (HC), raw black tiger prawn (RP) and heated black tiger prawn (HP) for 24 shellfish-allergic subjects. The cut-off of two standard deviations above mean reactivity of 7 non-atopic subjects to each of the extracts is indicated by the dotted line (0.56 for RC, 0.49 for HC, 0.29 for RP and 0.75 for HP).

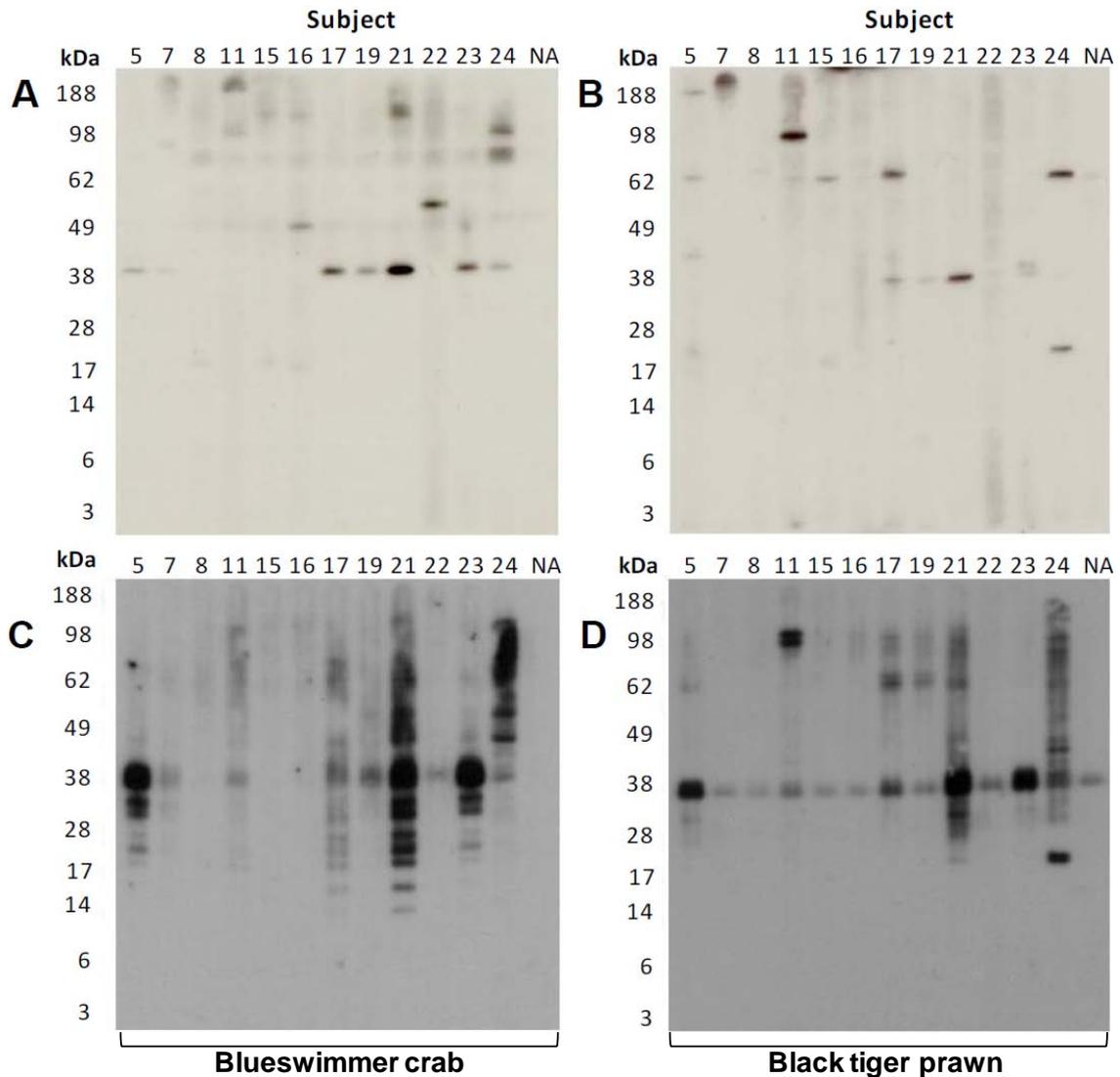
### 6.4.3 IgE Immunoblotting

Sera from 12 subjects with IgE positivity to RC and/or HC by ELISA, and where sufficient serum was available, were used for immunoblotting to visualise IgE-reactive proteins in the shellfish extracts (Figure 6.3). Immunoblotting showed markedly increased IgE binding to heated compared with raw extracts, in terms of number of proteins recognised and intensity of binding. In particular there was increased IgE binding to proteins within the TM region (37-39 kDa); 9 (75%) subjects showed IgE binding within this region for HC (7 in RC) and 7 (58%) subjects for HP (3 in RP). The identity of the protein(s) within this region was confirmed as TM using an anti-TM mAb. An increase in IgE-reactive lower molecular weight proteins (<39 kDa) was observed in the HC extract and to a lesser extent in the HP extract. A protein of about 62 kDa was recognised by 5/12 (42%) subjects in the RP extract but showed little or no reactivity in the crab raw or heated extracts. For both the raw and heated extracts, IgE-reactive proteins were observed at 40 kDa and 20-28 kDa corresponding to the documented allergens arginine kinase, and sarcoplasmic calcium binding protein, myosin light chain and troponin C, respectively.

### 6.4.4 Sequence analysis of blue swimmer crab tropomyosin

Following the analysis of allergenic proteins using IgE immunoblot, the highly IgE-reactive 39 kDa protein of the blue swimmer crab was identified as TM by peptide mass fingerprinting analysis (Table 6.2). The blue swimmer crab TM was subsequently cloned and the complete sequence derived from cDNA and published in Genbank under accession number JX874982 (Figure 6.4). This allergen has been designated *Por p 1* by the IUIS allergen nomenclature subcommittee (<http://www.allergen.org/index.php>). The most similar TMs were from the American lobster (*Homarus americanus*) and the black tiger prawn (*Penaeus monodon*) which both showed 98 % sequence identity. Sequence identity with other *Portunus* species TM, and the only crab TM, Cha f 1, listed within the IUIS allergen database was 92%. There were no amino acid differences between relevant regions in the blue swimmer crab TM and published linear IgE epitopes described for *Penaeus aztecus* (*Pen a 1*)<sup>21, 22</sup> and *Penaeus monodon* (*Pen m 1*)<sup>23</sup> TMs, except for the last epitope (amino acid

266 to 280) where two amino acid substitutions were identified. Another region (amino acid 43 to 56) of known and predicted IgE epitope specificity for other crustacean species showed several amino acid differences for the blue swimmer crab (Figure 6.4).



**Figure 6.3:** Immunoblotting for serum IgE reactivity against shellfish extracts. Sera from 12 shellfish-allergic subjects (5-24) and one non-atopic (NA) subject were tested for IgE reactivity to shellfish extract proteins separated by 4-12% SDS-PAGE. A. RC, B. RP C. HC, D. HP.

**Table 6.2:** List of generated peptides of blue swimmer crab tropomyosin extracted from SDS-PAGE gel using trypsin digestion and mass spectroscopy analysis

Residue numbers *	Peptide sequence
77-90	ALQNAEGEVAALNR
92-101	IQLLEEDLER
141-149	MDALENQLK
168-178	KLAMVEADLER
190-198	IVELEEEELR
252-264	EVDRLDEDELVNEK

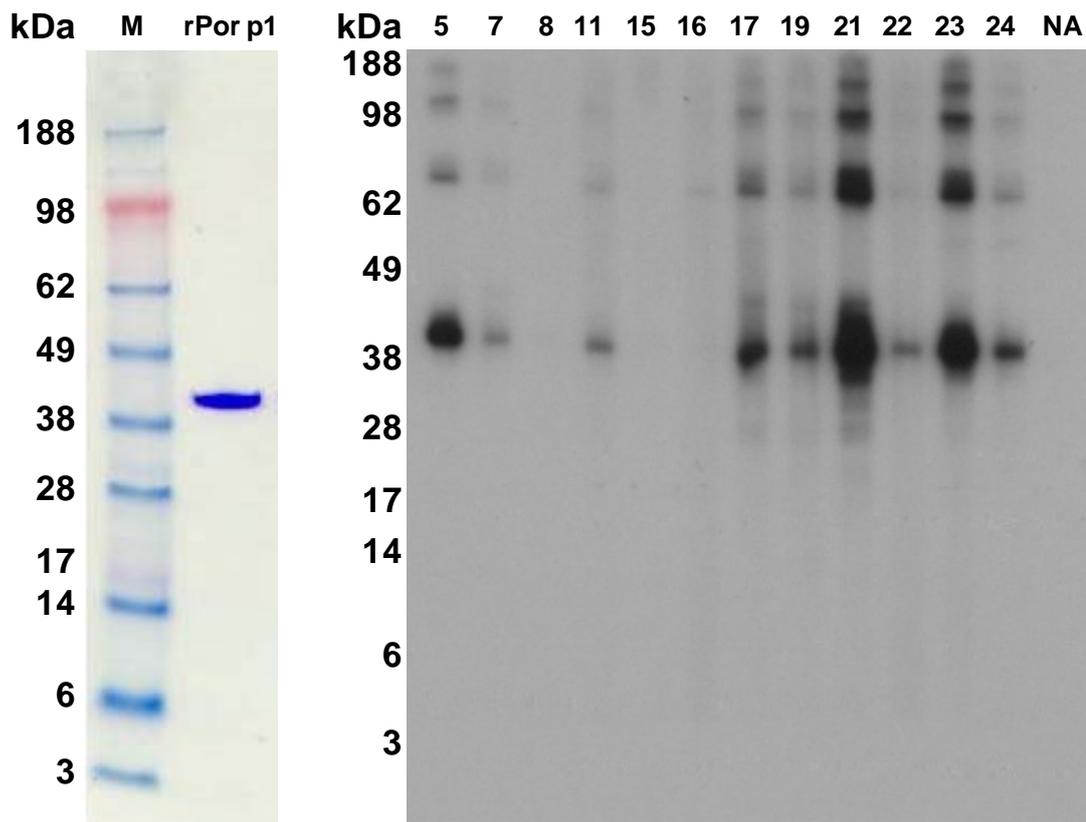
\* Residue numbers corresponding to the full sequence of blue swimmer crab tropomyosin, *Por p 1* deduced by cDNA analysis (Genbank accession number JX874982)

#### 6.4.5 IgE reactivity of recombinant crab tropomyosin, *Por p 1*

Recombinant crab tropomyosin was successfully expressed and purified with approximately 95% purity. In denaturing conditions, crab rTM was visible as a single band with a molecular weight of approximately 42 kDa (Figure 6.5A). The additional molecular weight as compared to the natural crab TM was due to the linker chain and the 6x histidine amino acid chain attached to the recombinant crab TM. Immunoblotting using sera from allergic patients revealed strong IgE binding to the 42 kDa recombinant crab tropomyosin in 75% of the patients (Figure 6.5B). Moreover, IgE bands were observed at approximately 75 kDa, which could be a possible dimeric form of the recombinant tropomyosin. Subsequently, based on the allergen sequence analysis and patient IgE binding data, blueswimmer crab tropomyosin was submitted as a new characterised allergen with the International Union of Immunological Studies (IUIS) Allergen nomenclature database as *Por p 1*.

<i>Portunus pelagicus</i>	1	MDAIKKKMQA	MKLEKDDAMD	RADTLEQQNK	EANIRA EKAE	EEVHNLQKRM	QQLENDLDQV
<i>Penaeus monodon</i>	1	-----	-----N-----	-----	-----N-----S-	-----	-----
<i>Penaeus aztecus</i>	1	-----	-----N-----	-----	-----N-----S-	-----	-----
<i>Portunus trituberculatus</i>	1	-----	-----N-----	-----N-----	-----L-----T-	IRAT--K-	--V--E--A
<i>Portunus sanguinolentus</i>	1	-----	-----N-----	-----	-----L-----T-	IRAT--K-	--V--E--A
<i>Charybdis feriatus</i>	1	-----	-----N-----	-----	-----L-----T-	IRAT--K-	--V--E--A
<i>Homarus americanus</i>	1	-----	-----N-----	-----	-----S-----	-----	-----
<i>Portunus pelagicus</i>	61	QESLLKANTQ	LEEKDKALSN	AEGEVAALNR	RIQLLEEDLE	RSEERLNTAT	TKLAEASQAA
<i>Penaeus monodon</i>	61	-----I-	-V-----	-----	-----	-----	-----
<i>Penaeus aztecus</i>	61	-----I-	-V-----	-----	-----	-----	-----
<i>Portunus trituberculatus</i>	61	--Q-SA--K	-D-E--Q-	-----	-----	-----	-----
<i>Portunus sanguinolentus</i>	61	--Q-SA--K	-D-E--Q-	-----	-----	-----	-----
<i>Charybdis feriatus</i>	61	--Q-SA--K	-D-E--Q-	-----	-----P-----	-----	-----
<i>Homarus americanus</i>	61	-----	-----	-----	-----	-----	-----
<i>Portunus pelagicus</i>	121	DESERMVKVL	ENRSLSD EER	MDALENQLKE	ARFLAEEADR	KYDEVARKLA	MVEADLERAE
<i>Penaeus monodon</i>	121	-----	-----	-----	-----	-----	-----
<i>Penaeus aztecus</i>	121	-----	-----	-----	-----	-----	-----
<i>Portunus trituberculatus</i>	121	-----	-----	-----	-----	-----	-----
<i>Portunus sanguinolentus</i>	121	-----	-----	-----	-----	-----	-----
<i>Charybdis feriatus</i>	121	-----	-----	-----	-----	-----	-----
<i>Homarus americanus</i>	121	-----	-----	-----	-----	-----	-----
<i>Portunus pelagicus</i>	181	ERAETGESKI	VELEEEELRVV	GNNLKSLEVS	EKANQREEA	YKEQIKTLTN	KLKAAEARAE
<i>Penaeus monodon</i>	181	-----	-----	-----	-----	-----	-----
<i>Penaeus aztecus</i>	181	-----	-----	-----	-----	-----	-----
<i>Portunus trituberculatus</i>	181	-----	-----	-----	-----	-----	-----
<i>Portunus sanguinolentus</i>	181	-----	-----	-----	-----	-----	-----
<i>Charybdis feriatus</i>	181	-----S-----	-----	-----	-----	-----T-----	-----A-----
<i>Homarus americanus</i>	181	-----	-----	-----	-----	-----	-----A-----
<i>Portunus pelagicus</i>	241	FAERSVQKIQ	KEVDRLEDEL	VNEKEKYKST	TDELDQAFSE	LSGY	
<i>Penaeus monodon</i>	241	-----	-----	-----I-----	-----T-----	-----	-----
<i>Penaeus aztecus</i>	241	-----	-----	-----I-----	-----T-----	-----	-----
<i>Portunus trituberculatus</i>	241	-----	-----	-----I-----	-----T-----	-----	-----
<i>Portunus sanguinolentus</i>	241	-----	-----	-----I-----	-----T-----	-----	-----
<i>Charybdis feriatus</i>	241	-----	-----	-----	-----	-----	-----
<i>Homarus americanus</i>	241	-----	-----	-----I-----	-----T-----	-----	-----

**Figure 6.4:** Tropomyosin sequence alignment. Alignment of tropomyosin sequences with *Portunus pelagicus* (blue swimmer crab) tropomyosin, *Por p 1* (Genbank accession number JX874982), as reference using NCBI Protein BLAST. Species include *Penaeus monodon* (NCBI protein database accession number: A1KYZ2.1), *Portunus trituberculatus* (ABL89183.1), *Portunus sanguinolentus* (ABS12234.1), *Charybdis feriatus* (Q9N2R3.1) and *Homarus americanus* (AAC48288.1). Sequences that correlate with known linear IgE binding epitopes of *Penaeus aztecus* tropomyosin (*Pen a 1*)<sup>21, 22</sup> are boxed. Predicted linear IgE epitopes based on studies with *Penaeus monodon* tropomyosin (*Pen m 1*)<sup>23</sup> are shaded grey.

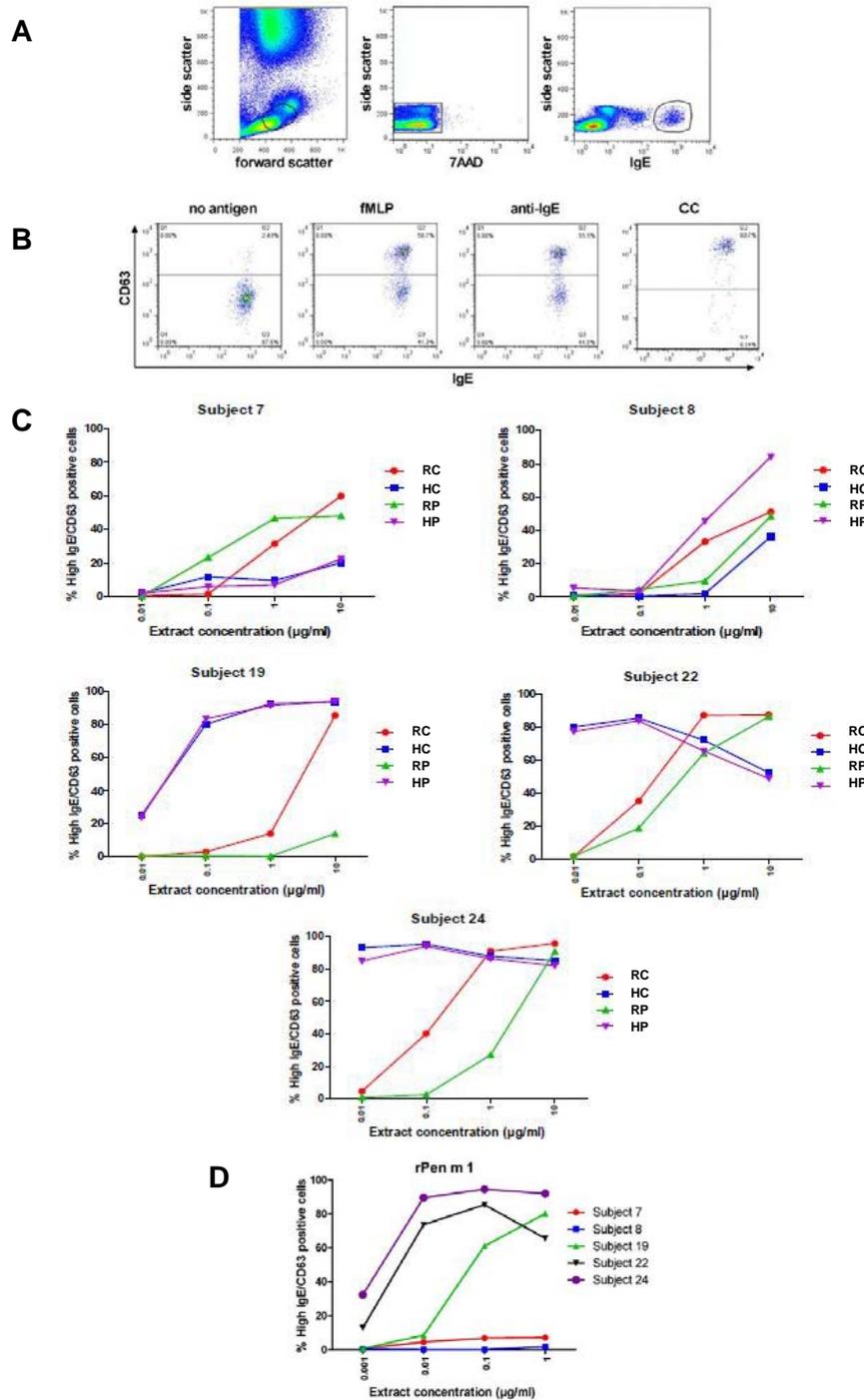


**Figure 6.5:** Characterisation of recombinant blue swimmer crab tropomyosin, *rPor p1*. A. 4-12% SDS-PAGE of recombinant blue swimmer crab TM, *rPor p1* stained with coomassie brilliant blue. M, MW markers. B. Sera from 12 shellfish-allergic subjects (5-24) and one non-atopic (NA) subject were tested for IgE reactivity to *rPor p1* using direct IgE immunoblotting.

#### 6.4.6 Basophil Activation Test

To assess biologically relevant shellfish allergen-specific IgE antibody reactivity, the ability of the different extracts to activate basophils from five shellfish-allergic subjects (7, 8, 19, 22, 24) was analysed by flow cytometry. Activated basophils were identified by high IgE expression and up-regulation of surface CD63 (Figure 6.6 A, B). No non-allergen specific activation of basophils or toxicity was caused by the different shellfish extracts, as determined by incubating extracts with the basophils from a non-atopic subject.

Dose-dependent basophil activation to the crab and prawn extracts was observed, with a range of sensitivities for the subjects, consistent with their different crab- and prawn-specific IgE reactivities by ImmunoCAP and our ELISA and immunoblotting assays (Figure 6.6 C). When subject basophil sensitivities were compared by examining the extract concentration required for 50% maximal stimulation, three subjects (19, 22, 24) showed markedly higher basophil activation by the heated extracts than the raw extracts, with little difference between the two crustacean species. Subjects 7 and 8 showed lower basophil activation with similar sensitivity to the four extracts. *rPen m1* was shown to induce strong basophil activation in those subjects with high reactivity to the whole extracts (Figure 6.6 D). Interestingly, *rPen m1* did not induce basophil activation in subject 7 and 8, which did not elicit reactivity to the heated shellfish extracts but to raw extracts. This strongly indicates sensitisation and IgE reactivity to heat-labile shellfish allergens and not to tropomyosin in these subjects. This highlights the importance of testing against raw shellfish extracts in addition to heated extracts for improved diagnosis.



**Figure 6.6:** *In vitro* basophil activation by shellfish extracts and *rPen m1*. A. Representative dot plots showing gating of viable basophils (7AAD, high IgE positive). B. Representative dot plots (subject 19) showing analysis of activated basophils (up-regulation of cell surface CD63) for negative and positive controls, and HC (10 µg/mL). C. Dose-dependent activation of basophils from shellfish-allergic subjects (7, 8, 19, 22, 24) by shellfish extracts. D. Dose-dependent activation of basophils from shellfish-allergic subjects (7, 8, 19, 22, 24) by *rPen m1*.

### 6.4.7 Inhibition IgE ELISA

Inhibition ELISA was used to quantitate the degree of IgE cross-reactivity between the two shellfish species and the effects of cooking. No non-specific inhibition of IgE reactivity by the shellfish extracts or *rPen m 1* was evident using serum IgE from a Bahia grass pollen-sensitised control subject and Bahia grass pollen extract. Using shellfish-allergic subjects, *rPen m 1* inhibited > 50% serum IgE reactivity to HC and HP at the lowest concentration (0.048 µg/mL) for all except two subjects (Table 6.3). For these two exceptions (11, 24), marked IgE reactivity to allergens other than TM was supported by immunoblot data. Both heated extracts showed greater inhibition of IgE binding than the corresponding raw extracts. For most subjects, the lowest concentration of heated extract (0.16 µg/mL) resulted in > 50% inhibition of IgE binding to both HC and HP. RC was a more efficient inhibitor of IgE binding to the heated extracts than RP.

**Table 6.3:** Inhibitor concentration required for 50% inhibition of IgE binding to heated blue swimmer crab (HC) extract or heated black tiger prawn (HP) extract

Subject ID	Coating antigen: HC					Coating antigen: HP				
	Inhibitor					Inhibitor				
	RC	HC	RP	HP	<i>rPen m 1</i>	RC	HC	RP	HP	<i>rPen m 1</i>
5	12	†	*	†	†	60	0.64	*	†	†
11	2.4	†	16	†	1.2	18	†	20	†	1.2
17	1.3	†	36	†	†	1.6	†	47	†	†
19	0.8	†	60	†	†	2.4	†	47	†	†
21	1.3	†	47	†	†	2.9	†	47	†	†
23	4	†	87	†	†	20	†	90	†	†
24	*	14	84	20	0.14	2.1	†	20	†	†

\* - did not reach 50% inhibition at the maximum inhibitor concentration of 100 µg/mL  
 † - did not drop below 50% inhibition at the minimum inhibitor concentration of 0.16 µg/mL (0.048 µg/mL for *rPen m 1*)

## 6.5 Discussion

Crustaceans, especially crabs and prawns, are a common cause of shellfish allergy world-wide. This study examined the IgE-reactive components of a commonly eaten crustacean species, the blue swimmer crab, compared with those of a well characterised species, the Black tiger prawn. In particular, the effects of heating on IgE reactivity and cross-reactivity of the crab allergens were investigated.

When serum IgE reactivity of shellfish-allergic subjects to whole extracts was compared, the raw crab extract elicited greater IgE reactivity when compared with the raw prawn extract by both ELISA and immunoblotting. Whether this was due to inherent differences between the proteins of the blue swimmer crab and Black tiger prawn, or to differences in sensitising species or route of sensitisation is not clear. It has been shown previously that inhalation during commercial processing can result in sensitisation to seafood.<sup>24-28</sup> In the present study, although some subjects identified food handling as a cause of adverse reaction, this route could not be distinguished in all subjects and the majority reported ingestion-related allergic episodes. More strikingly, it was found that heated extracts were more IgE reactive than raw for both species. This may be due to the more common ingestion of heated crab and prawn or to chemical modification of the crustacean proteins during heating as discussed below. That the IgE reactivity of the crustacean extracts observed in this study was clinically relevant was demonstrated by the functional, basophil activation test, again confirming higher allergenicity of the heated extracts.

IgE immunoblotting studies were performed to examine individual IgE-reactive proteins. As for other crustacean species, tropomyosin (TM) is a major allergen of the blue swimmer crab. Over 50% of shellfish-allergic subjects showed IgE-reactivity to proteins corresponding to TM in both raw and heated crab extracts and TM identity was confirmed by TM-specific mAb reactivity and peptide mass fingerprinting of the highly IgE-reactive 39 kDa protein. This study reports for the first time, the cloning and full sequence analysis of the *Portunus pelagicus* TM, *Por p 1*. This revealed strong homology of the blue swimmer crab TM with other crustacean TMs, but regions of amino acid sequence difference at sites of

known and predicted linear IgE epitopes support the need for crustacean species-specific diagnostic reagents. Several other allergenic proteins of the blue swimmer crab were recognised at lower frequency. For some subjects, these proteins were recognised in the absence of TM reactivity. In particular, the inhibition studies suggested that an IgE-reactive protein at 78 kDa in the heated crab extract may be a unique (as yet uncharacterised) allergen as it showed limited cross-reactivity with Black tiger prawn. In Chapter 4, a 70 kDa IgE binding protein was identified as a fragment of the protein titin. This 78 kDa band may be a similar muscle protein present in the blue swimmer crab. Testing of a larger subject cohort is required to assess clinical importance of the unique and shared allergens observed in our study and hence define an appropriate panel of defined allergens for refined diagnostic assays.

In view of potential food matrix-associated effects on the outcome of heating of allergens<sup>27</sup>, heating of whole extracts rather than purified allergens was chosen for further examination. For both the crab and prawn species studied, ELISA and immunoblotting showed markedly increased IgE reactivity of heated extracts compared with raw. In particular, IgE immunoblotting demonstrated increased IgE-reactivity of TM within the heated extracts. A range of highly IgE-reactive lower molecular weight proteins was also observed following heating, presumably largely TM fragments since this was especially notable for sera that reacted with the single TM band in the raw extracts. Moreover this phenomena of IgE binding allergen fragment was demonstrated for Black tiger prawn tropomyosin in Chapter 4. Investigation of the mechanisms responsible for the increase in allergenicity of TM and other allergens within the whole shellfish extracts following cooking is warranted as most shellfish are heat processed before consumption. Potential mechanisms include denaturation of protein with exposure of neo-epitopes and the Maillard reaction. In this heat-dependent reaction, sugars, both endogenous and exogenous, are non-enzymatically attached at different locations on the protein molecule generating advanced glycation end products.<sup>18, 29</sup> The Maillard reaction has not been well explored in the context of shellfish allergy, although found to play a role in the IgE reactivity of other allergens, particularly peanut allergens.<sup>30, 31</sup> The findings of this study

support the inclusion of thermally-processed whole extract as well as defined allergen preparations in commercial diagnostic tests for shellfish allergy.

Cross-reactivity between crustacean species is essential to understand in order for shellfish-allergic subjects to receive the best clinical advice on food avoidance. In this study, it was shown that IgE cross-reactivity between the blue swimmer crab and Black tiger prawn was high, especially between the heated extracts. Cross-reactivity between the heated extracts was symmetric as both were able to effectively inhibit IgE binding to each other to a similar extent.<sup>32</sup> This means that the sensitising species is unable to be determined in most cases without accurate clinical history. As shown by inhibition ELISA, 50% inhibition of IgE binding to the heated crab extract by *rPen m 1.0101* and heated whole extract inhibitors was achieved at similar concentrations, consistent with allergenicity in the heated blue swimmer crab largely due to cross-reactive TM. TM has previously been documented as the major allergen of the Black tiger prawn.<sup>3, 4</sup> The sequence analysis of the Blue swimmer crab TM, *Por p 1*, and alignment with Black tiger prawn TM, *Pen m 1.0101*, provides a molecular basis for the high IgE cross-reactivity observed between these species in this study.

Screening of shellfish-allergic subjects by IgE ELISA against raw and heated extracts gave insight into whether the current diagnostic ImmunoCAP for crab is relevant in a southern hemisphere clinical setting. Although a double-blind placebo-controlled food challenge can confirm diagnosis of food allergy, for adults with shellfish allergy this procedure has a high risk of anaphylaxis and is not routinely performed.<sup>33</sup> For this reason, in this study only ImmunoCAP data together with a careful clinical history were collected. In most cases, subjects were unable to identify clearly which individual crustacean species had provoked their clinical reaction. However, for the three subjects who did identify crab as an offending species, only two of these had positive ImmunoCAP scores for crab-specific IgE. The third subject (No.14) had a negative crab ImmunoCAP but tested positive for both raw and heated blue swimmer crab in our IgE ELISA. A significant correlation was found between the heated, but not raw, crustacean extract ELISA results and ImmunoCAP scores, but in addition to the subject mentioned above, a small number of subjects with a negative

crab ImmunoCAP result showed IgE reactivity to the crab extracts in both the IgE ELISA and immunoblot. This finding suggests either greater sensitivity of our assays, and/or lack of appropriate crab species or preparation method in the ImmunoCAP allergen preparation (*Cancer pagurus* or brown crab, a northern hemisphere species). All subjects with IgE-reactivity to crab or prawn TM by immunoblot had moderate to high levels of allergen-specific IgE ( $\geq 2.37$  kU/L) as determined by ImmunoCAP to shrimp and/or crab. These subjects were also more likely to have had severe allergic symptoms, such as angioedema and anaphylaxis, upon contact with shellfish. However, there were some subjects who had a strong ImmunoCAP result to shrimp and/or crab and clinical history of severe reactions but showed low or no IgE reactivity by ELISA or immunoblot. These subjects may have species-specific IgE with limited or no cross-reactivity with the crustacean species in this study<sup>34, 35</sup>, likely due to the ingestion of different species.

Previous studies have concluded that specific-IgE to TM is an accurate predictor of shrimp allergy.<sup>36, 37</sup> This chapter supports the importance of TM as a major allergen of the blue swimmer crab, but several other crab proteins were shown to be allergenic and subjects exhibited different allergen reactivity profiles, several with no TM IgE reactivity. Component-resolved allergen-microarray technology would allow the simultaneous screening of serum IgE reactivity to a full panel of shellfish allergens, including whole allergen extracts, purified native and recombinant allergens, allergen fragments, and heated and raw preparations. This would be of great advantage for the sensitive and specific diagnosis of shellfish allergy, and more information regarding the correlation between allergen-sensitisation and severity of clinical symptoms could be gathered.

In conclusion, heating causes a marked increase in clinically relevant IgE reactivity of blue swimmer crab extract. In particular, the blue swimmer crab TM, *Por p 1*, was identified and characterised as a heat-stable major cross-reactive allergen. Other IgE-reactive blue swimmer crab proteins were observed, some corresponding to molecular weights of documented shellfish allergens, but others currently unidentified, some potentially unique to the blue

swimmer crab. The findings of this chapter will advance reliable diagnosis and management of shellfish allergy.

In Chapters 4, 5 and 6, the diagnostic and clinical aspects of shellfish allergy were investigated, novel allergens identified and characterised and effects of heat processing investigated. Another important aspect of shellfish allergy is the exposure and sensitisation due to air-borne allergens, which may lead to asthma and may eventually induce allergic sensitisation. The next chapter demonstrates the detection of air-borne tropomyosin, which affects shellfish processing workers in the crab industry. Detection of aerosolised allergens in different working activities will help in better management and reduction of air-borne particle generation and improve work-safety and occupational health.

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## 6.7 Chapter 6 summary

- Current clinical allergy diagnostic platforms do not consider the various crab species commonly consumed in the southern hemisphere and the effect of heat processing, which may lead to false negative diagnosis.
- This study examined the effects of heating on blue swimmer crab (*Portunus pelagicus*) allergens in comparison with those of Black tiger prawn (*Penaeus monodon*) by testing reactivity with shellfish-allergic subjects' serum IgE.
- Cooked extracts of both species showed markedly increased IgE reactivity by ELISA and immunoblotting, and clinical relevance of IgE reactivity was confirmed by basophil activation tests.
- The major blue swimmer crab allergen tropomyosin, *Por p 1*, was cloned and sequenced, showing strong homology with tropomyosin of other crustacean species but also sequence variation within known and predicted linear IgE epitopes.

## CHAPTER 7

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### MOLECULAR AND IMMUNOLOGICAL APPROACHES IN QUANTIFYING THE AIR-BORNE FOOD ALLERGEN – TROPOMYOSIN

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## 7.1 Introduction

Occupational allergy and asthma has become a serious health concern, especially for workers in the seafood industry. Increased global consumption and changing dietary habits have greatly facilitated seafood production.<sup>1,2</sup> This in turn, has caused more workers to be exposed to seafood allergens on a daily basis. According to a report by the Food and Agriculture Organization (FAO) in 2010, nearly 45 million people are involved in seafood and aquaculture production. Several studies have shown that the prevalence of occupational asthma among workers exposed to shellfish is between 4% to 36%.<sup>3-5</sup> Moreover, workers with occupational asthma to shellfish were shown to develop ingestion-related food allergies to the same shellfish species.<sup>6</sup> Occupational exposure to shellfish allergens can elicit upper and lower respiratory tract symptoms such as asthma, rhinitis and also cause skin symptoms.<sup>1,7,8</sup>

In the seafood industry, workers are constantly exposed to air-borne shellfish particulate matter arising from the different processing techniques. Several studies have shown the presence of allergenic proteins in air-borne particulate matter, which are responsible for causing allergic sensitisation among affected workers.<sup>9-11</sup> Serum IgE antibody reactivity to allergen among workers due to occupational exposure of crab matter has been reported previously.<sup>12-16</sup> Aerosolisation of shellfish allergens occurs due to processes such as filleting, freezing, cooking, smoking, drying and techniques using high pressure water/air.<sup>1,6</sup> Processes such as butchering, de-gilling and boiling particularly, have been shown to cause excessive bioaerosol formation. The wet or dry air-borne particles may then be inhaled by exposed workers. Abdel Rahman *et.al*, demonstrated elevated levels of air-borne crab allergens in specific work stations such as butchering and cooking as compared to cleaning, packing and storage.<sup>17</sup>

As described in Chapter 2, the two groups; crustaceans (shrimps, crabs, lobsters) and molluscs (oyster, mussels, octopus, squid) together are commonly termed as shellfish.<sup>18, 19</sup> Subsequently in Chapter 4, it was demonstrated that the major shellfish allergen tropomyosin displays a

remarkable stability to heating and is able to retain its allergenicity even in heat-processed shellfish products.<sup>20, 21</sup>

As shown in Chapter 5, shellfish tropomyosin belongs to a family of highly conserved proteins. More importantly, the conserved nature of tropomyosin is responsible for the allergic cross-sensitisation among various invertebrates such as crustaceans, molluscs, mites and insects. A case report in 2002 described the occurrence of eczema on hands after handling shrimps by a Chinese cook with crustacean-mite syndrome.<sup>22</sup> A study in 2003 by Fernandes *et al.* has demonstrated the cross-sensitisation to shrimps in an unexposed Jewish population with dust mite allergy.<sup>23</sup>

Antibody reactivity to tropomyosin is a good predictor of shellfish allergy as demonstrated in Chapter 4 and several other studies.<sup>21, 24, 25</sup> Due to its excellent structural stability and detailed characterisation, tropomyosin was chosen as an ideal molecular marker for detecting air-borne shellfish allergens in this study.

This chapter describes the development and validation of a highly sensitive immunoassay to detect and quantify aerosolised tropomyosin in air samples collected from two crab processing factories. Using this immunoassay, air-borne tropomyosin was detected and quantified in a worker- and activity-specific manner. The approach of using a recombinant protein as standard and purified natural allergen to generate the capture antibody for increased sensitivity and specificity has not been employed previously. This methodology can be modified for the quantification of other major food allergens and would be an important tool in monitoring air-borne allergen levels in different work environments. This can subsequently assist in establishing safety paradigms to control the unintentional generation of aerosolised allergens and accidental sensitisation of exposed workers.

## 7.2 Aims

The specific aims for the work described in this chapter were

- To develop and validate an antibody-based immunoassay for the specific and sensitive quantification of the major crustacean allergen tropomyosin in aerosolised form.
- To quantify air-borne crab tropomyosin in the breathing zones of 80 workers in a crab processing environment using the developed immunoassay.

## 7.3 Materials and Methods

### 7.3.1 Allergen standard: Expression and purification of recombinant tropomyosin

Recombinant tropomyosin was expressed and purified as described previously in Chapter 2.<sup>21</sup> Briefly, total RNA was extracted from fresh specimens of black tiger prawn (*Penaeus monodon*) using the RNeasy mini extraction kit (Qiagen, Hilden Germany) according to the manufacturer's instructions. Complementary DNA (cDNA) was reverse transcribed from the total RNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Basel, Switzerland), following the manufacturer's instructions. The coding region for tropomyosin was amplified by PCR using forward 5'-GCGGATCCGACGCCATCAAGAAGAAGATGC-3' and reverse 5'-GCGAATTCTTAGTAGCCAGACAGTTCGCTG-3' primers. The PCR conditions were set as follows, 94 °C for 2 min, 30 cycles of 94 °C for 20 sec, 55 °C for 20 sec, 72 °C for 30 sec and a final elongation step, 72 °C for 7min. The 860bp amplified product was cloned into the expression vector pRSET-A using the *Bam*H1 and *Eco*R1 restriction sites. The recombinant expression vector, pRSET-A-TM was transformed into BL21 *Escherichia coli* strain and expression of the recombinant tropomyosin with a HIS-tag, induced using 1 mM Isopropyl  $\beta$ -D-1-thiogalactopyranoside (Amresco, USA). The bacterial cells were washed with extraction buffer (25 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM imidazole) and lysed using a French pressure cell. After centrifugation at 6000 g for 15 minutes, the recombinant tropomyosin was purified using HIS-Trap FF Affinity Column (GE Healthcare, USA). The fraction containing the recombinant protein was further purified using a Superdex G75 16/600 size exclusion column (GE Healthcare, USA) on a Biologic Duoflow FPLC (BioRad, Hercules, CA, USA). The purified recombinant tropomyosin was labelled "rTm" and stored in aliquots at - 80°C until further use.

### 7.3.2 Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described in chapter 4 to confirm the purity of the tropomyosin standard and analyse the binding characteristics of the purified antibodies. Twelve micrograms of the protein samples was heated in Laemmli buffer containing dithiothreitol and loaded onto a 12% bis-acrylamide gel. Protein separation was performed at 180V using a Mini-Protean Tetra Cell electrophoresis system (BioRad, Hercules, CA, USA). The separated proteins were visualised by staining with Coomassie brilliant blue R250 (BioRad, Hercules, CA, USA).

### 7.3.3 CD Spectroscopy of allergen standard

Circular Dichroism spectroscopy was performed to analyse the alpha helical content of rTM and compare it to purified natural prawn tropomyosin. Natural and recombinant tropomyosin samples were prepared in PBS, pH 7.2 and adjusted to a final concentration of 3  $\mu$ M. CD spectroscopy was performed on a J715 Spectropolarimeter (Jasco, USA) with continuous nitrogen flushing at 25°C. All measurements were performed using a 10mm quartz cuvette over a wavelength range of 190-260 nm. For wavelength analysis, the tropomyosin samples were scanned with a step width of 0.2 nm and bandwidth of 1nm at 100nm/min averaging over eight scans. Final data was expressed as mean residual ellipticity ( $\Theta$ ) after subtracting PBS blank spectrum.

### 7.3.4 Production of polyclonal anti-tropomyosin antibodies

#### 7.3.4.1 Protein extraction and estimation

To prepare the antigen mixture to generate antibodies in rabbits, protein extracts were generated from four crustacean species; Black tiger prawn (*Penaeus monodon*), Vannamei prawn (*Litopenaeus vannamei*), Banana prawn (*Fenneropenaeus merguensis*) and School prawn (*Metapenaeus macleayi*) as

described in chapter 2.<sup>21</sup> Briefly, the complete shellfish specimen, in its outer shell, was heated in liquid (PBS) at 100°C for 20 minutes. The outer shell of the specimen was then removed and the edible tissue cut into small pieces. Fifty grams of the muscle mass was homogenised in 150 mL of phosphate buffered saline (PBS) for 10 minutes using an Ultra turrax blender (IKA, Staufen, Germany), agitated for 3 hours at 4°C followed by centrifugation at 14,000 rpm for 15 minutes. The supernatant was clarified through a glass fibre filter, followed by filtration through a 0.45 µm membrane filter (Millipore, Billerica, MA, USA) and stored at -80°C until further use.

To characterise the generated polyclonal antibody, unheated protein extracts were prepared from crab (*Portunus pelagicus*), lobster (*Jasus edwardsii*), fish (*Lates calcarifer*) and pork (*Sus scrofa*) as described elsewhere.<sup>20</sup>

#### **7.3.4.2 Tropomyosin-Antigen mix preparation**

The protein extracts produced in section 7.3.4.1 were used as starting material to purify tropomyosin. Ten milligrams of the protein extract was loaded on a mini Macro-prep High Q strong anion exchange column, (Bio Rad, Hercules, CA, USA) in 30 mM tris-HCl, pH 6.5. Tropomyosin was eluted from the column using a linear gradient with increasing sodium chloride salt concentration in tris-HCl buffer from 0.4 M to 0.6 M, over 20 column volumes. The purified TM fractions were pooled and a buffer exchange with phosphate buffered saline (PBS) performed using Amikon spin filters with a 3kDa molecular weight cut-off (Millipore, Billerica, MA, USA). The tropomyosin fractions from the four crustacean species were mixed and adjusted to a final concentration of 1 mg/mL in sterile PBS

#### **7.3.4.3 Rabbit immunization**

The immunisation of rabbits was performed at IMVS, Adelaide, Australia. New Zealand rabbits were injected with the prepared tropomyosin immunogen along with Freund's adjuvant in four doses at two week intervals. The pre-bleed was

collected at week 0 to serve as a negative control. A test bleed was collected at week 7 to test the production of the antibodies and the final bleed conducted at week 9. The collected serum was stored at  $-80^{\circ}\text{C}$  until further processing.

#### **7.3.4.4 Enrichment and purification of polyclonal IgG anti-tropomyosin antibodies (Capture antibodies)**

For the development of the sandwich ELISA, IgG antibodies were enriched from the rabbit serum using sodium sulphate precipitation. Sodium sulphate (Sigma Aldrich, USA) was added to 10 mL of serum in small quantities at a time, to a final concentration of 18% w/v. The serum was then centrifuged at 1500g for 10 min at ambient temperature, the pellet resuspended in 18% w/v sodium sulphate solution, re-centrifuged and finally re-dissolved in 5 mL of sterile PBS, pH 7.2. The IgG enriched serum fraction was subsequently dialysed against PBS overnight and then stored at  $-80^{\circ}\text{C}$  until further use.

The Aminolink Plus Immobilisation kit (Thermo Scientific, Melbourne, Australia) was used to prepare a tropomyosin affinity column for the isolation and purification of tropomyosin specific IgG antibodies from the IgG enriched rabbit serum fraction. The procedure was followed according to the manufacturer's instructions. Briefly, rTm was covalently bound to the column, the remaining active sites blocked and washed, and finally stored in PBS, pH 7.2. The IgG enriched rabbit serum fraction was loaded onto the tropomyosin affinity column and incubated for 30 minutes. The anti-tropomyosin IgG antibody fraction was subsequently eluted using 0.2M glycine hydrochloride, pH 2.5 and neutralised with 1 M tris hydrochloride, pH 8.5. The purified antibody fraction was then dialysed against PBS, pH 7.2 and stored in aliquots with 0.05% sodium azide at  $-20^{\circ}\text{C}$  for further use. This fraction was labelled cAb- $\alpha\text{TM}$ .

#### **7.3.4.5 Biotinylation of detection antibodies**

A fraction of the anti-tropomyosin IgG antibodies were biotinylated using the EZ-Link® Sulfo-NHS-LC-Biotin (Thermo Fischer Scientific, Melbourne,

Australia) following the manufacturer's instructions. A 100 fold molar excess of biotin was used to biotinylate the antibodies from a freshly prepared 10 mM biotin stock solution. After biotinylation, the antibody solution was dialysed against PBS, pH 7.4 to remove the reactants. Sodium azide was added to the antibody solution to a final concentration of 0.05% w/v and stored at 4°C in amber coloured tubes and labelled dAb- $\alpha$ TM.

### 7.3.5 Immunoblotting

IgE immunoblotting was performed to test patient IgE reactivity of rTm as described in Chapter 4.<sup>26</sup> To analyse the binding characteristics of the capture antibody to various antigenic sources, the separated proteins on an SDS-PAGE gel were transferred to a polyvinylidene fluoride (PVDF) membrane using the Semi-dry TransBlot Apparatus (BioRad, Hercules, CA, USA). After blocking with 1% (w/v) bovine serum albumin (BSA) in PBS-T, the membrane was subsequently incubated with the capture antibody, cAb- $\alpha$ TM, diluted 1:10,000 in 0.5% BSA, PBS-T and goat anti-rabbit IgG antibody conjugated with HRP (Promega, USA) diluted 1:20,000. Antibody binding was visualised using the enhanced chemiluminescent technique as previously reported.<sup>10</sup>

### 7.3.6 Tropomyosin amino acid sequence alignment

To predict the capture antibody binding characteristics to crustacean tropomyosin, a protein sequence alignment was performed to compare vertebrate and invertebrate tropomyosin. cDNA based protein sequences for tropomyosin were collected from the NCBI database with the following accession numbers; Black tiger prawn, accession number (AAX37288.1), Vannamei prawn (ACB38288.1), King crab (BAF47266.1), Snow crab (A2V735.1), Rock lobster (AFY98827.1), American lobster (O44119.1), Cod fish (BAC44994.1) and Pig tropomyosin (NP\_001090952.1). Sequence alignment, matrix identity scores and percent similarity were calculated using Clustal O algorithm in Jalview program.<sup>27</sup> The evolutionary history was inferred using the Minimum Evolution method. The tree is drawn to scale, with branch lengths in

the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm at a search level of 1. The analysis involved 7 amino acid sequences. There were a total of 284 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

### **7.3.7 Air sampling and elution of aerosolised allergen, tropomyosin**

Collection of aerosolised tropomyosin was performed using polytetrafluoroethylene (PTFE) filters with a pore size of 1.0  $\mu\text{m}$  (Millipore, Billerica, USA). The filter cassette apparatus was attached to a pump (SKC Ltd, UK) through a tube and the airflow adjusted to 2.5 L/min. The average airflow rate through the filter, was calculated as the mean of initial and final airflow at the start and stop of the pump, respectively. Where a change in the airflow from start to finish was more than 10%, the samples were discarded.

The air-collection apparatus was setup in a backpack, which was carried by each worker during their normal shift. The air collection inlet was placed in the workers personal breathing zone (PBZ) so as to sample the air available for breathing.

Elution of the collected allergens was performed using Nunc-Immuno Minisorp tubes (Nunc, USA) to minimise the loss of allergen content due to adsorption on the tube walls. The PTFE filters were removed from the cassettes and placed in tubes containing 1 mL of phosphate buffered saline (PBS) with 0.5% Tween 20 and 0.2 mM sodium azide. The extraction was performed, by placing the tubes on a rotation tilter for 2 hours at room temperature. The eluate was transferred to a new Minisorp tube and bovine serum albumin (BSA) added to a final concentration of 1 mg/mL. This eluate was then aliquoted in 200  $\mu\text{L}$  volume and stored in mini eppendorf tubes at  $-80^{\circ}\text{C}$  until further analysis. A clean unused filter was eluted simultaneously using the extraction procedure above and regarded as a blank sample.

### 7.3.8 Assay procedure

All incubations were performed with 100  $\mu$ L/well at room temperature for 1 hour unless otherwise stated. All washing steps were performed using PBS with 0.05% v/v tween 20, pH7.2 (PBS-T) and repeated three times on an EL405 Autoplate washer (BioTek Instruments, Winooski, USA) unless otherwise stated. A 96 well high binding Costar microtitre plate (Sigma Aldrich, USA) was coated with cAb- $\alpha$ TM diluted 1:500 in carbonate buffer, pH 9.6 and incubated overnight at 4°C. After washing, the wells were blocked with 270  $\mu$ L of Pierce Superblock buffer (Thermo Fischer Scientific, Melbourne, Australia). Next either the standards serially diluted from 10 to 0.02 ng/mL, or the test air samples or test blanks were added to the wells and incubated for 3 hours. After washing, the detection antibody dAb- $\alpha$ TM diluted 1:500 in dilution buffer (PBS-T containing 1 mg/mL BSA) was added to the wells. Subsequently, the streptavidin-horse radish peroxidase conjugate (Sigma Aldrich, USA) diluted 1:10,000 in dilution buffer was added to the wells and incubated for 30 minutes. The wells were then washed five times and patted dry. To visualise antibody binding, 100  $\mu$ L of 3,3',5,5'-tetramethylbenzidine substrate (Becton Dickinson, USA) was added to the wells until a blue coloration started forming in the blank wells and the reaction stopped using 1N hydrochloric acid. The colour development in the wells was measured at 450 nm using a Versamax Microplate Reader (Molecular Devices, California, USA).

### 7.3.9 Validation of sandwich ELISA

#### 7.3.9.1 Linearity of the standard curve, calculation of Limit of Detection (LOD) and Limit of Quantitation (LOQ) and non-specificity

The validity of the rTm standard curve was assessed using the  $R^2$  value based on a four-parameter logistic curve calculated using SoftMax Pro software v5.2 (Molecular Devices, California, USA). Non-specific binding of the assay was analysed by omitting the capture antibody, cAb- $\alpha$ TM or the detection antibody, dAb- $\alpha$ TM. TM levels in the air collection samples were derived by interpolation of the absorbance readings of the rTm standard curve using four parametric

logistic algorithm. The air collection samples were diluted in the range of 1:2 to 1:80 to obtain an absorbance value within the linear range of the standard curve. The allergen standard curve was included in every 96 well plate used to analyse the test samples.

To test the sensitivity of this assay, blank samples were run in 12 separate experiments in triplicates. The limit of detection (LOD) was calculated as the mean of the blank samples plus three times the standard deviation. The limit of quantitation (LOQ) was calculated as the mean of the blank samples plus ten times the standard deviation.<sup>28</sup>

### 7.3.9.2 Spike recovery assays

Spiking tests were performed to test the matrix interference effects of the extraction buffer and other extraneous air-borne entities in the actual air sample. Seventeen random air-collection samples were selected with either low levels of tropomyosin or with levels below the LOQ, and spiked with rTm. An equal volume of test samples and rTm (spike) were mixed to a final concentration of 1 ng/mL. These spiked samples were then frozen at -80°C overnight to simulate the air collection sample preparation process. The next day, the spiked samples were thawed to room temperature and tested using the immunoassay as described in section 7.3.8. Percent recovery (%) was derived by dividing the measured TM concentration of the spiked sample by the sum of TM concentration of un-spiked sample and the spike concentration; [spiked sample (ng/mL)/(un-spiked sample (ng/mL)+Spike, 1ng/mL)]. The recovery rates of the sample had to fall within 70 -110% for the assay to pass.<sup>29</sup>

### 7.3.9.3 Precision of the ELISA

The precision of this sandwich ELISA was tested on the basis of intra-assay and inter-assay variability. Air-collection test samples with tropomyosin levels below the LOQ were pooled and diluted 1:2 in extraction buffer to be spiked. Three spiked samples were prepared at low (0.2 ng/mL), medium (0.5 ng/mL)

and high (1.0 ng/mL) concentrations of rTm. For intra-assay variability tests, 9 replicates were tested for each spike concentrations (low, medium and high) in one single 96 well plate which included a standard curve. To test the inter-assay variability, the 3 spiked samples were tested in six different experiments over three days by two independent operators. Each test sample was tested in triplicates and each experiment included a standard curve. For the assay to pass, the Co-efficient of variation (% CV) of the replicates had to fall within 20%, for both intra-assay and inter-assay tests. In addition, the percent recovery had to fall within 20% of the theoretical concentration.<sup>30</sup>

### **7.3.10 Statistical analysis**

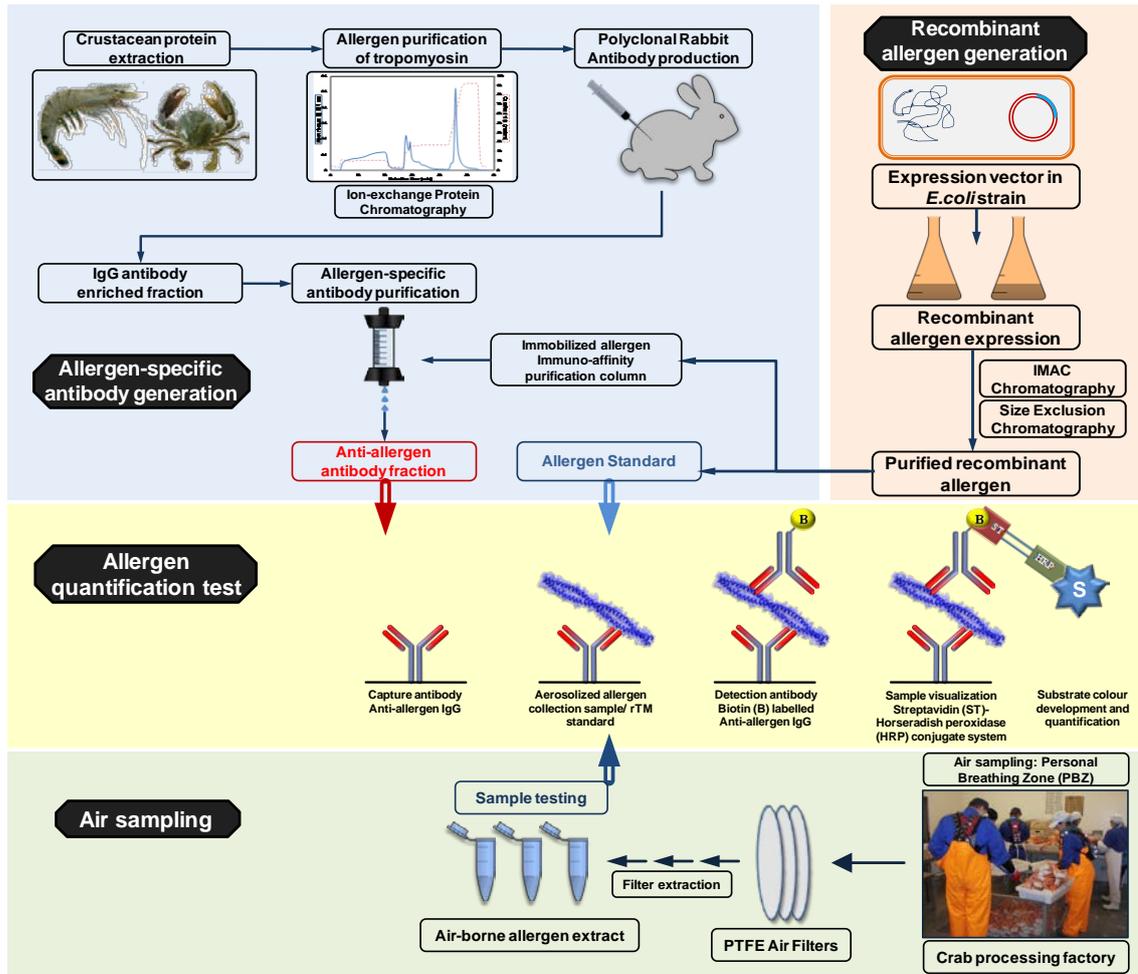
The Mann Whitney U Test was used to compare the allergen exposure levels in each category of work-tasks. A p value of less than 0.05 was considered significant. All statistical analysis was performed using GraphPad Prism version 6.02 (GraphPad, USA).

## 7.4 Results

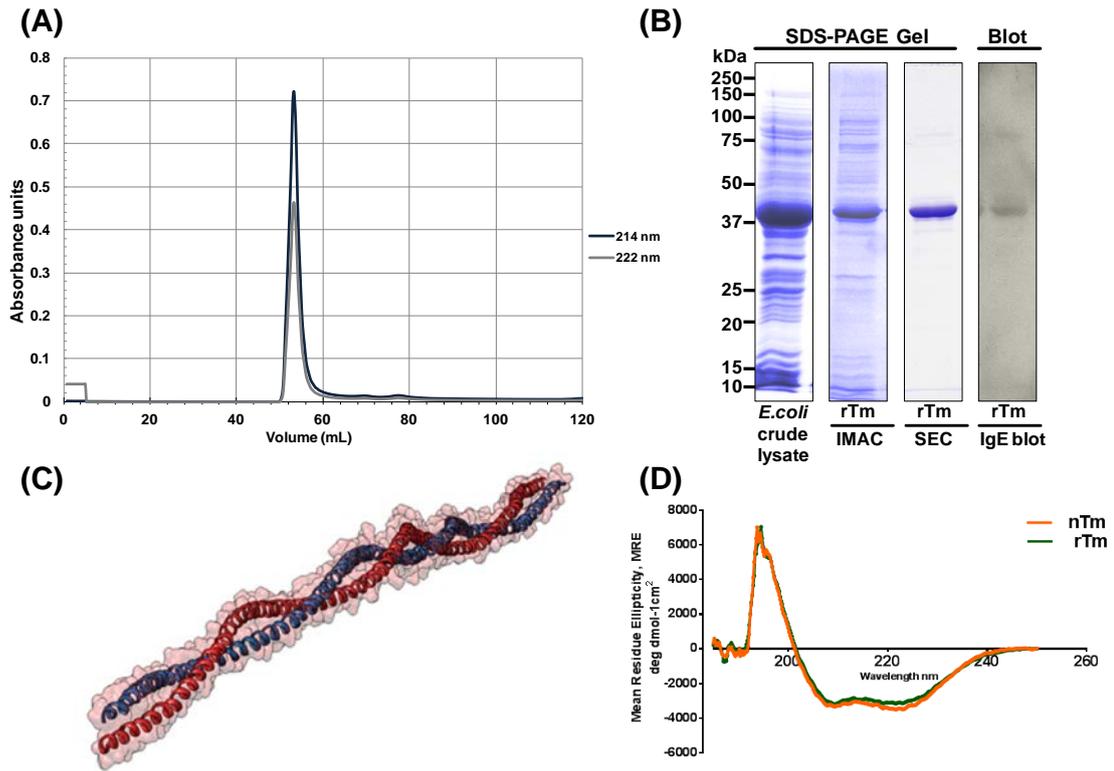
### 7.4.1 Characterisation of the allergen standard (rTm)

The allergen standard, rTm was successfully expressed in a BL21 *E.coli* bacterial expression system as represented in Figure 7.1. rTm was expressed as a fusion protein with a 6X histidine-tag at the N-terminal end of the protein to facilitate purification. Affinity chromatography was performed to purify rTm from the crude bacterial lysate. However, several additional bands could be observed in the affinity purified fraction (Figure 7.2 A). Therefore, size exclusion chromatography (SEC) was subsequently performed as an additional purification step. The final product was visible as a single band of approximately 40 kDa. To demonstrate immunological reactivity, immunoblotting experiments were performed (Figure 7.2 B). Immunoblotting with patient sera confirmed IgE antibody reactivity and thus the allergenicity of rTm. Protein homology modelling of the allergen tropomyosin represents its highly stable alpha-helical structure and the favourable formation of a homo-dimeric state (Figure 7.2 C).

To confirm appropriate protein folding and structure of the recombinant protein and its subsequent use as an allergen standard, CD spectroscopic analysis of rTm and natural tropomyosin was performed (Figure 7.2 D). A distinct negative signal at 208 and 222 nm is typical for an alpha helical protein. This was also observed for the rTm as compared to the purified natural tropomyosin. The CD spectrum for rTm was almost identical to that of natural tropomyosin.



**Figure 7.1:** Schematic representation of the methodology and setup used for the detection and quantification of the air-borne shellfish allergen tropomyosin



**Figure 7.2:** Characterisation of the immunoassay allergen standard, recombinant tropomyosin, rTm. (A) Protein purification profile of rTm using size exclusion chromatography, (B) SDS-PAGE analysis of the various purification stages of the allergen standard using nickel affinity (IMAC), size exclusion chromatography (SEC) and IgE Immunoblotting analysis of rTm using pooled patient sera, (C) Three dimensional homology model of crustacean tropomyosin in dimeric form. (D) Comparison of alpha-helical content of allergen standard, rTm (green) and natural tropomyosin, nTm (orange) using CD spectroscopy

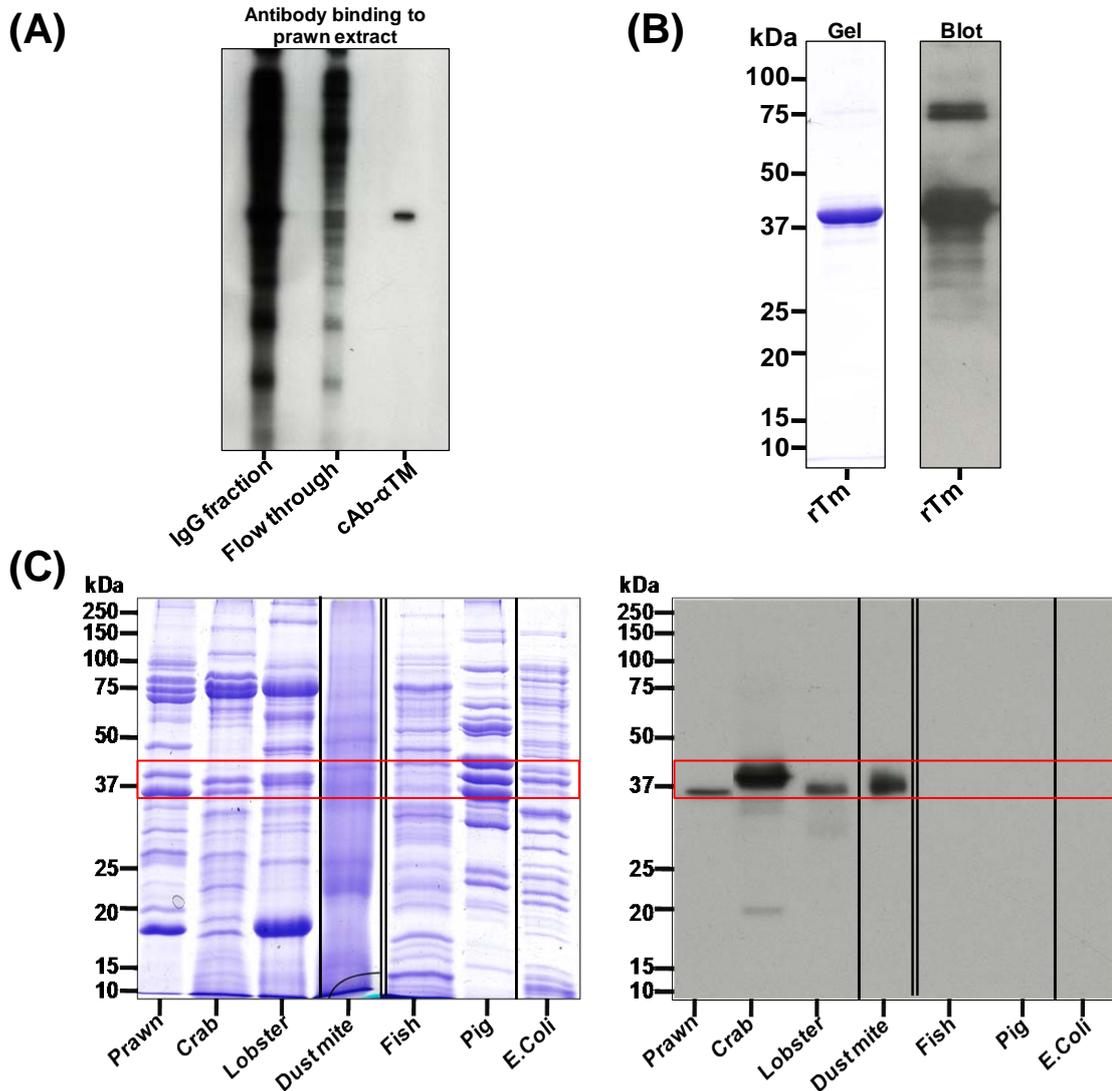
### 7.4.2 Binding properties of the capture antibody

The polyclonal anti-tropomyosin antibody, cAb- $\alpha$ TM was successfully isolated from the IgG enriched fraction of the immunised rabbit serum as shown in Figure 7.1. Immunoblotting demonstrated specific binding of cAb- $\alpha$ TM to a 37 kDa band from prawn crude extract (Figure 7.3 A). Specific antibody reactivity to tropomyosin was confirmed with strong binding to rTm (Figure 7.3 B). Moreover, antibody reactivity was observed to the dimeric form of tropomyosin formed at 75 kDa. The antibody binding characteristics of cAb- $\alpha$ TM was analysed against various antigenic sources. cAb- $\alpha$ TM showed strong binding to tropomyosin from the crustaceans analysed; crab, prawn and lobster. In contrast, no binding was observed to extracts from fish, chicken and *E.coli* (Figure 7.3 C). Interestingly, antibody binding was observed to tropomyosin from house dust mite extract at 37 kDa region. Thus, antibody specificity was demonstrated to invertebrate tropomyosin.

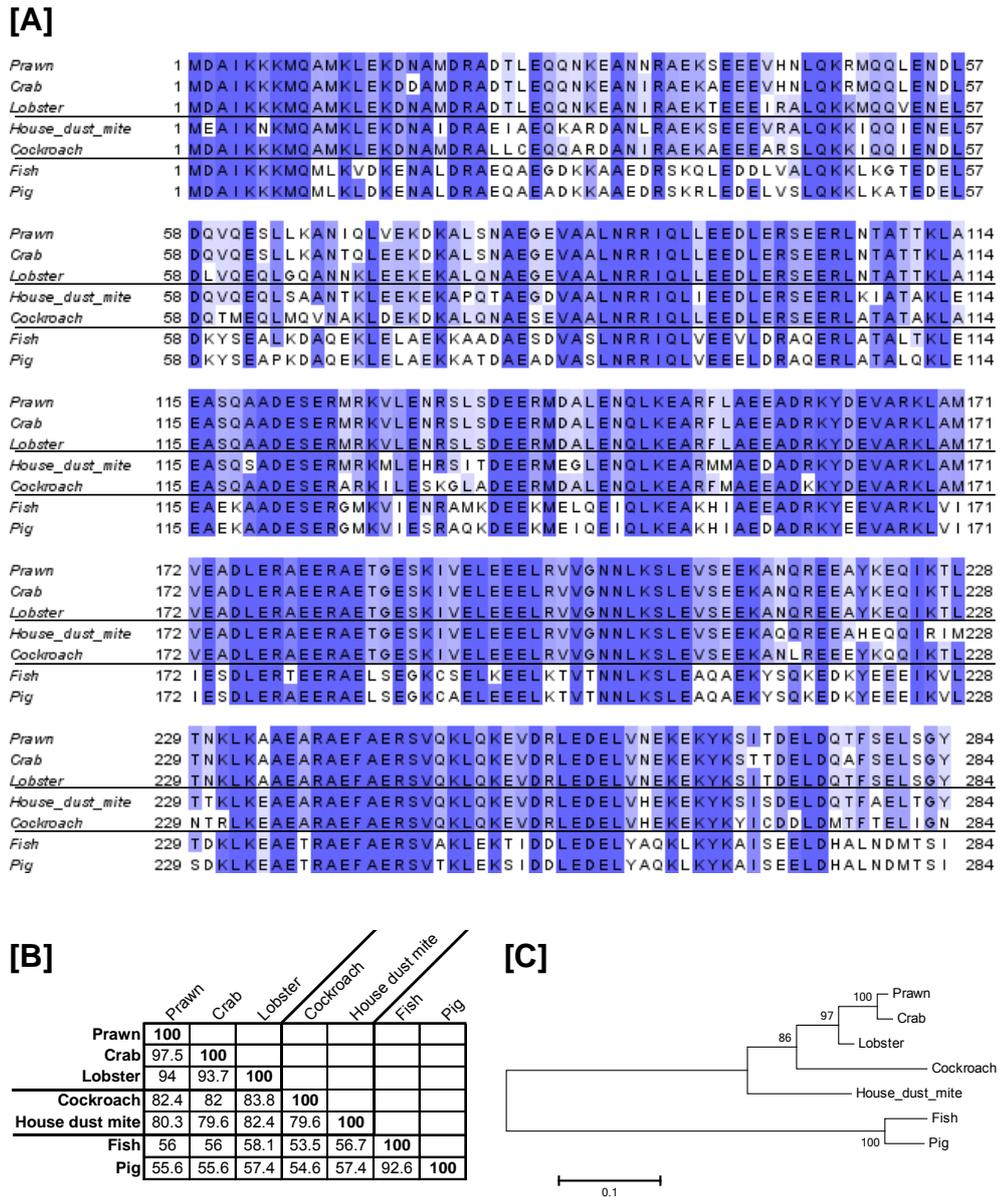
Selective antibody binding to crustacean tropomyosin may be attributed to molecular differences in the primary structure of tropomyosin among vertebrates and invertebrates (Figure 7.4). The compared crustacean tropomyosin was at least 94% identical among each other. However, when compared to vertebrate tropomyosin, the maximum percent identity was only 58% (Figure 7.4B). Cockroach and house dust mite tropomyosin, both of which have been characterised as allergens, were closely related to crustacean tropomyosin with 79-82% sequence identity (Figure 7.4B, 4C).

### 7.4.3 Linearity of allergen standard curve

A ten point serial dilution curve of rTm was used in the range of 0.02 to 10 ng/mL concentration, diluted using air sample extraction buffer. A standard curve was generated using the absolute allergen concentrations in ng/mL and its corresponding absorbance values measured at 450 nm using a four-parameter logistic regression algorithm. Error bars represent the standard deviation. The goodness of fit ( $R^2$ ) was 0.998 averaged from six individual experiments (Figure 7.5 A). The linear region of the standard curve, 0.02 ng/mL to 1.25 ng/mL was used for the quantification of tropomyosin in the air collection samples.



**Figure 7.3:** Binding characteristics of polyclonal rabbit anti-tropomyosin IgG antibody. (A) Antibody binding patterns of various stages of rabbit antibody purification against prawn protein extract. (B) SDS-PAGE and immunoblotting of the allergen standard rTm using cAb- $\alpha$ TM antibody. (C) SDS-PAGE profiles of different invertebrate and vertebrate species extract and specific antibody binding of cAb- $\alpha$ TM against different protein extracts. *E.coli* extract was used a recombinant protein expression control.

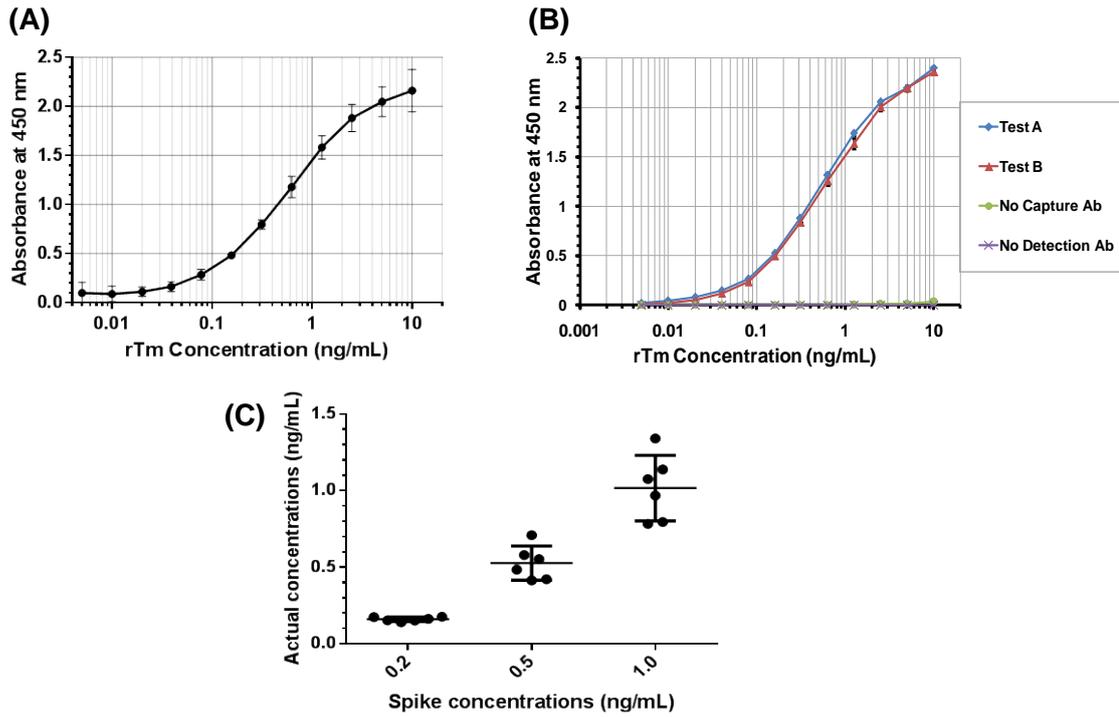


**Figure 7.4:** Comparison of Invertebrate tropomyosin primary structure. [A] Amino acid sequence alignment, for visual comparison of primary structure similarity of invertebrate and vertebrate tropomyosins. Accession number (Genbank nucleotide sequence database) Prawn, *Penaeus monodon*, accession number (AAX37288.1); Crab, *Portunus pelagicus*, (BAF47266.1); Lobster, *Jasus lalandii* (AFY98827.1); House dust mite, *Dermatophagoides pteronyssinus*, (ACI32128.1); Cockroach, *Blattella germanica*, (AAF72534.1); Fish, *Gadus chalcogrammus*, (BAC44994.1); and Pig, *Sus scrofa* tropomyosin (NP\_001090952.1). The colored shading intensity indicates percent conservation (Dark – 100%, Medium – 12 to 30%, Light – <50%). [B] Tropomyosin amino acid sequence identity table calculated using Clustal Omega. [C] Phylogenetic tree of invertebrate tropomyosin inferred using the minimum evolution method.

To quantify tropomyosin in the air collection samples were diluted from 1/2 to 1/80 to bring the absorbance values within the linear range of the standard curve. This allowed for accurate measurement of samples with very high or low allergen content. Use of higher concentrations of rTm, beyond 2.5 ng/mL, did not result in a proportional increase in the absorbance values (Figure 7.5 B). The limit of detection (LOD) was calculated to be 60 pg/mL and the limit of quantitation (LOQ) was determined to be 100 pg/mL.

#### 7.4.4 Assay specificity

The accuracy of an immunoassay depends on the absence of non-specific antibody binding. This can be attributed to specific non-specific binding and non-specific non specific binding.<sup>30</sup> The cAb- $\alpha$ TM was tested for specific non-specific binding and as shown in Figure 7.3; no binding was observed to any other protein but tropomyosin. Subsequently, the assay was conducted with the entire standard curve by omitting the capture antibody, cAb- $\alpha$ TM or the detection antibody. This confirmed the specificity of this assay to rTm with absence of non-specific binding to other reagents, since a complete loss of signal was observed (Figure 7.5 A). Finally, the spike recovery test was performed to analyse the interference from non-related matrix agents present in the air-borne particulate matter collected in the air samples (Table 7.1). Results are shown as percent recovery of the rTm spike (0.5 ng/mL) in blank samples and test samples with tropomyosin content below the assay LOQ. The mean recovery of the spike was calculated to be  $78.8\% \pm 6.5\%$  which passed our pre-set criteria.<sup>30-32</sup> Moreover, there were no cases of positive interference among control samples. Two of 17 samples had a recovery below 70% suggesting possible interference with the sample matrix.



**Figure 7.5:** Standard curve for the allergen standard using recombinant tropomyosin (rTm). (A) A 10 point serial dilution curve from 10 to 0.02 ng/mL, error bars indicate the standard deviation of each dilution over six individual experiments. (B) Assay reproducibility and specificity: Test A and B indicate the standard curves from two separate experiments. ● and X indicate omission of the capture or detection antibodies respectively to analyse non-specific binding properties of the immunoassay. (C) Inter-assay variability test using rTm spiked samples at three different concentration; 0.2, 0.5 and 1.0 ng/mL

**Table 7.1:** Matrix interference and spike recovery analysis of the Tm sandwich ELISA. Spike recovery assay was performed on 17 random air collection samples by spiking with 0.5 ng/mL of rTm standard. Percent recovery (%) was calculated by comparing the theoretical concentration and detected concentration of rTm in ng/mL. Mean recovery was calculated as 78.8%  $\pm$  6.5%.

Sample no.	Unspiked	Spiked samples with	Recovery of spike
	Air-collection samples	rTm (0.5 ng/mL)	Tm
	Tm (ng/mL)	Tm (ng/mL)	Percent (%)
1	<LOQ	0.36	71.0
2	<LOQ	0.39	78.0
3	<LOQ	0.40	80.0
4	<LOQ	0.34	67.6
5	<LOQ	0.43	86.0
6	<LOQ	0.41	81.2
7	<LOQ	0.36	71.6
8	<LOQ	0.40	79.0
9	<LOQ	0.34	68.4
10	<LOQ	0.46	91.8
11	<LOQ	0.40	79.4
12	0.69	0.94	78.9
13	0.31	0.64	79.6
14	0.36	0.71	83.2
15	0.89	1.24	89.2
16	0.29	0.60	76.2
17	0.49	0.78	79.0

#### 7.4.5 Intra-assay and inter-assay variability

Intra- and inter-assay variability tests were conducted to test the precision and accuracy of the assay (Table 7.2). Variability was tested for low, medium and high concentration of tropomyosin (Figure 7.5 C). The mean recoveries of the samples were over 70% and the co-efficient of variation was <20% for all three concentrations of rTm tested. This data are in concordance with the acceptance criteria for assay validation.<sup>30-32</sup>

#### 7.4.6 Analysis of air samples from crab processing factory

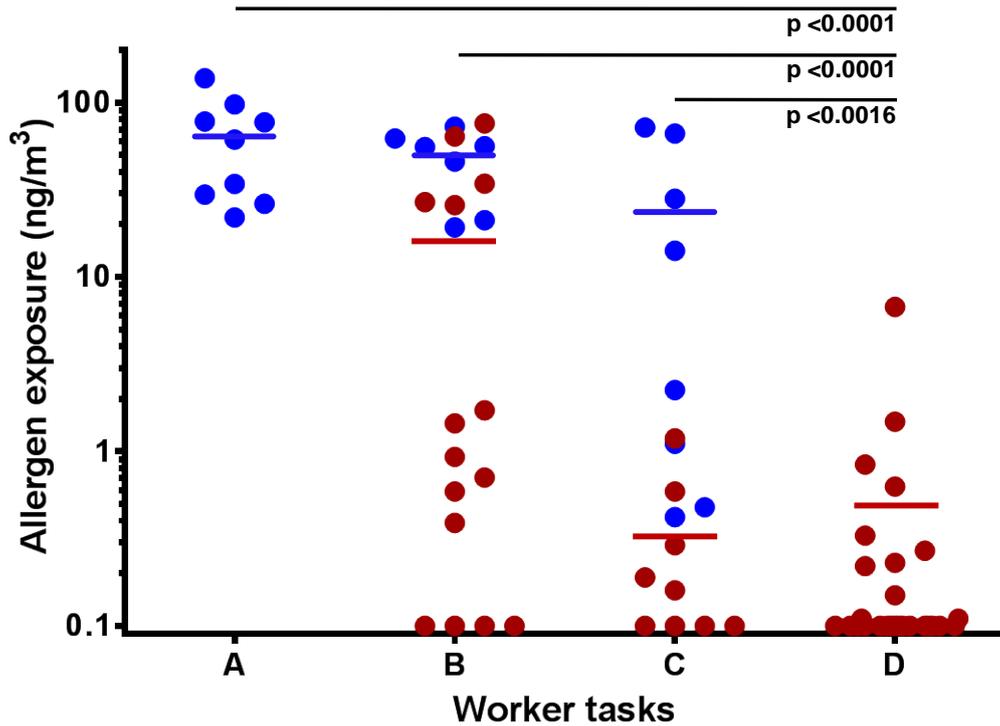
Air-samples were tested from the PBZs of workers from two different processing activities; king crab and edible crab (Table 7.3). The average air volume sampled was  $1095 \pm 118$  L and  $830 \pm 371$  L for edible crab and king crab processing, respectively. The amount of air-borne tropomyosin and exposure patterns differed among the two processing plants (Figure 7.6). Tropomyosin in the king crab plant measured in the range of 0.15 to 75.89 ng/m<sup>3</sup> whereas in the edible crab, it was 0.42 to 138 ng/m<sup>3</sup>.

In the edible crab processing, the highest tropomyosin exposure was demonstrated for workers handling boiled meat and spinceller (separator of meat from boiled crab). The lowest exposure was in the scanning process, freezer and raw crab handling area. The tropomyosin exposure concentrations varied significantly among workers in the high exposure activities, handling boiled crab.

King crab processing was performed in batches interspersed with fish processing, due to the seasonal availability of the crab. The highest tropomyosin concentrations were identified for processes such as cleaning, cracking and crab degilling. Among workers sharing work tasks between crab and fish processing, moderate concentrations of tropomyosin exposure was observed. Area locations and tasks such as truck driving, fish packing, receiving stations and fish gutting did not show any significant concentrations of air-borne tropomyosin.

**Table 7.2:** Assay precision of the immunoassay. Intra- and Inter-assay variability of the assay was tested using 0.2 ng/mL, 0.5 ng/mL and 1 ng/mL of rTm as low, medium and high antigen concentrations, respectively. The assay variability is shown in terms of the calculated mean concentration, standard deviation and percentage of the coefficient of variation. Recovery of the rTm concentrations were calculated as a percentage of the ratio of calculated versus theoretical concentrations

<b>Intra-assay variability</b>				
<b>rTm (ng/mL)</b>	<b>Mean (ng/mL)</b>	<b>Standard deviation, SD</b>	<b>Coefficient of variation, CV (%)</b>	<b>Recovery (%)</b>
0.2	0.14	0.013	9	70.5
0.5	0.40	0.025	6.3	79.8
1.0	0.76	0.074	9.8	76.4
<b>Inter-assay variability</b>				
<b>rTm (ng/mL)</b>	<b>Mean (ng/mL)</b>	<b>Standard deviation, SD</b>	<b>Coefficient of variation, CV (%)</b>	<b>Recovery (%)</b>
0.2	0.16	0.018	11.0	80.0
0.5	0.41	0.043	10.4	82.0
1.0	0.81	0.074	9.1	81.0



**Figure 7.6:** Allergen exposure to crab processing workers involved in various tasks and locations. Allergen exposure to workers processing edible crabs is displayed as blue dots and king crabs in red dots. The mean exposure for each task category is shown as a horizontal line in blue and red for edible crab and king crab, respectively. Tasks; A, Handling boiled meat, B, Crab cracking, gutting, raw meat, cutting and cleaning, C, Crab receiving station, handling, scanning, sorting and packing, D, Logistics and fish-related tasks

**Table 7.3:** Exposure to air-borne allergen tropomyosin among workers in crab-processing workplace according to specific tasks performed

Cat.	Task function	Number of samples	Allergen exposure		
			Median (ng/m <sup>3</sup> )	Range (ng/m <sup>3</sup> )	p value*
A	handling boiled meat	9	61.4	21.95-138.8	<0.0001
	B	crab cracking	22	23.48	<LOQ-75.89
C	gutting	17	1.11	<LOQ-71.92	<0.0016
	raw meat handling				
	cutting				
	cleaning				
D	receiving station	27	0.25	<LOQ-6.74	---
	scanning				
	sorting				
	packing				
D	transport/ logistics	27	0.25	<LOQ-6.74	---
	fish-related tasks				

\* P values were calculated using the Mann–Whitney U test in comparison to category D.

## 7.5 Discussion

Frequent occurrences of allergic reactions among seafood workers due to air-borne allergen exposure have been reported.<sup>1</sup> Occupational asthma has been commonly associated with shellfish processing and previous studies have shown a prevalence of up to 36%.<sup>33</sup> A strong correlation between the high allergen exposure levels and development of allergenic sensitisation and asthma has been suggested.<sup>33, 34</sup> A number of tasks in the shellfish processing workplace put the workers at a greater risk of exposure and consequent sensitisation to shellfish allergens.<sup>1, 35</sup> Currently, there is a lack of standardised, validated methods for monitoring the allergen concentrations in bioaerosols produced during shellfish processing.

The aim of this study was to develop and validate a sensitive antibody-based immunoassay for the detection and quantification of the shellfish allergen tropomyosin in bioaerosols produced during crab processing.

Traditionally, serum IgE antibodies from shellfish-sensitised individuals have been used to detect air-borne allergens, using an inhibition ELISA setup.<sup>15, 16</sup> This approach is useful for quantifying air-borne IgE-reactive allergens, with the assay having a sensitive detection limit of 1ng/mL. However, the major disadvantage of IgE antibodies is their low titre and difficulty in developing a standard assay model due to varied antibody reactivity in different patient sera.

ELISA based immunoassays have been previously applied for the detection of tropomyosin in food matrices, using monoclonal or polyclonal antibody based platforms.<sup>36-38</sup> However, the assay sensitivity, precision and matrix interference levels varied largely among the different assays. Monoclonal antibody based assays have the advantage of high specificity and lack of false-positive results in allergen detection.<sup>39, 40</sup> Nevertheless, any modifications or changes in the conformation of the mAb-epitope on the allergenic protein may lead to loss of binding. Polyclonal antibody based assays on the other hand, can bind to the allergens at multiple epitopes, thus minimising the risk of protein conformational changes affecting the binding to the allergen.

In the present study, we have employed a modified approach for developing a highly sensitive immunoassay for the quantification of aerosolized crustacean allergens. One of the initial hurdles was to develop a polyclonal antibody based assay with high specificity and minimised unspecific binding. Natural tropomyosins purified from four different crustacean species were used for the generation of polyclonal rabbit antibodies. This increased the binding capacity of the antibody to tropomyosins with an amino acid sequence variation of over 95%. Moreover, a recombinant crustacean tropomyosin of high purity was used as the stationary phase for affinity purification of tropomyosin-specific IgG antibodies from the rabbit serum. The resultant final antibody fraction demonstrated high specificity to crustacean tropomyosin with no non-specific binding to other homologous proteins as shown in our validation tests.

Interestingly, the capture antibody was able to recognise house dust mite tropomyosin, due to the latter's high percent identity to crustacean tropomyosin. This however may not significantly affect the quantification of crab tropomyosin in a wet processing environment. Previous studies have shown that dust mites contain very low amounts of tropomyosin.<sup>41</sup> Moreover, it has been shown that tropomyosin may not be the main allergen involved in seafood-mite sensitisation.<sup>42</sup>

To improve the assay specificity, recombinant tropomyosin was used as an allergen standard in the assay to exactly quantify the amount of tropomyosin in the samples. The advantage of using a recombinant protein as a standard is the unlimited availability and consistent performance as opposed to the natural source, which often demonstrates batch to batch variations. Previous studies on allergen detection have developed assays with sensitivities ranging from 1 ng/m<sup>3</sup> to 105 ng/m<sup>3</sup>.<sup>15, 39</sup> The immunoassay developed in this study was able to achieve an allergen detection limit of 60 pg/m<sup>3</sup>. However, assays with higher sensitivity (10 pg/m<sup>3</sup>) have been developed for mouse or rat urinary allergens.<sup>43</sup>

The main parameters of performance of the assay were established by in-house validation. Spike recovery tests were performed to test the matrix interference effects and recorded as 79 %. While the outcome fell within the acceptance criteria, it highlighted the effects of matrix components on allergen-

antibody binding. This interference seems to result mainly from the extraction buffer used to elute the allergens from air sampling filters. The performance of the allergen standard curve was tested in the presence and absence of the buffer and showed a marked effect on the absorbance values of the allergen standard. Therefore, to maintain similar levels of matrix effects from the buffer on the test samples as well as the standards, the later was diluted using the same extraction buffer as for the sample analysis. The accuracy and precision (reproducibility) of the assay was tested using inter-assay and intra-assay variability tests. Both the tests met the acceptance criteria of falling within 20% co-efficient of variation.

This developed immunoassay was utilised to assess the exposure levels of tropomyosin in two different processing activities; edible crab and king crab. In general, the levels of bioaerosols and subsequently the levels of air-borne allergen concentrations are dependent on variables such as the kind of seafood being processed, the amount of seafood being processed, the size of the factory, layout of processing equipment and the locations of ventilation system. These variables vary with different locations and different seasons of seafood processing. For example, some king crab processing factories were mainly involved in fish processing and only temporarily converted to crab processing, depending on the availability and fishing season. Due to this, a small line of crab processing assembly is placed in relatively large production rooms where ventilation may not be optimal near the processing activity. Some of the edible crab processing activities involved cooking the crabs. This was performed in defined areas or separate rooms with point ventilations. Although measures were taken to limit the exposure to the fumes from the cooking vats, many workers not involved in the cooking activities were stationed close by and were exposed to the fumes. The edible crab processing facility was designed specifically for crab production and it involved workers standing close together on a processing line. In the king crab factories, the larger rooms and fewer workers involved in processing and more manual labour might account for some of the differences in allergen concentrations in the air samples tested, as compared to the edible crab factory.

Several shellfish allergens have been identified and characterised in commonly consumed shellfish species.<sup>19, 21, 26</sup> Tropomyosin, however is the most abundant and heat-stable invertebrate allergen inducing allergic sensitisation. In this study, the highest exposure to tropomyosin was demonstrated during heating and boiling processes as shown previously.<sup>15</sup> Recent studies have shown the ability of natural or heat generated tropomyosin fragments to elicit IgE reactivity and enhanced IgE reactivity of tropomyosin after heating.<sup>20, 21</sup> This is of clinical importance since the heating processes do not only increase the aerosolisation of allergens but can also exacerbate the IgE sensitisation among affected workers. From the detection point-of-view, it is an advantage using a polyclonal antibody based assay, since monoclonal based assays may not be able to detect these allergen fragments aerosolised during the heating or boiling processes.<sup>21</sup> The importance of allergen fragments is particularly important in the case of tropomyosin, as eight IgE binding epitopes have been discovered spanning its entire length of the alpha-helical secondary structure.<sup>19, 21, 44</sup> This may dramatically increase possible IgE cross-linking in sensitised individuals due to exposure to tropomyosin fragments.

In summary, a highly sensitive assay for air-borne tropomyosin was developed and validated. Using this assay, we were able to quantify tropomyosin in the PBZ of workers performing different work activities in crab processing factories. High concentrations of tropomyosin were detected mainly in the boiling, heating and de-gilling stations. The developed immunoassay is currently employed for monitoring allergen exposure in different crab processing factories as part of a larger work-safety study in Norway. The methodological approach for developing this assay offers opportunities to other industries to quantify air-borne food allergens to improve work-safety and occupational health.

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### 7.7 Chapter 7 summary

- Exposure and sensitisation to tropomyosin via inhalation is particularly important in the crustacean processing industry where workers are continuously exposed to the aerosolised form of this allergen.
- The aim of this study was to develop the first antibody-based immunoassay to enable the specific and sensitive quantification of aerosolised tropomyosin present in the environment of two crab processing facilities.
- Affinity purified polyclonal anti-tropomyosin antibodies were used as the allergen capture system and was able to selectively detect tropomyosin from different crustaceans, but not from fish or chicken (vertebrate tropomyosin).
- The novel immunoassay was able to successfully identify working activities which generate low, medium and high concentrations of the aerosolised food allergen.
- Heating or boiling of crab meat generate high levels of air-borne tropomyosin.

## CHAPTER 8

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### GENERAL DISCUSSION AND FUTURE DIRECTION

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Recent times have witnessed an increase in the number of cases of food allergies to shellfish. Currently, there is no cure for shellfish allergy and immunotherapeutic approaches have not yet been developed for desensitisation of the affected individuals. Current approaches for diagnosing shellfish allergy are based on empirical methods which may either entail a health risk to the patient (*in vivo* methods) or the possibility of false-negative results (*in vivo* or *in vitro* methods). This problem is further compounded by the diversity of consumed shellfish species around the world and particularly in the Asia-Pacific region, which has resulted in exposure and sensitisation to novel yet uncharacterised allergenic proteins. In addition, thermal processing of shellfish and its effects on the allergenicity and reactivity of allergens is not clearly understood.

The work outlined in this PhD thesis, aimed to characterise the novel allergens identified in commonly consumed shellfish species in the Asia-Pacific region. The major allergen, tropomyosin was identified and characterised from three different species; the Black tiger prawn (*Penaeus monodon*), King prawn (*Melicertus latisulcatus*) and Blue swimmer crab (*Portunus pelagicus*) in Chapter 4, Chapter 5 and Chapter 6 respectively. Furthermore, the characterised tropomyosins were registered as new major allergens, *Pen m 1*, *Mel l 1* and *Por p 1* respectively, with the IUIS Allergen database.

A comprehensive IgE-allergen binding analysis was performed for Black tiger prawn extract in Chapter 4 using advanced mass spectrometric techniques. Using this approach, six prawn allergens were identified, namely, arginine kinase, myosin light chain, sarcoplasmic calcium binding protein, triose-phosphate isomerase, including two putative novel allergens, fructose bisphosphate aldolase and titin fragment. For the first time, patient IgE binding to naturally-occurring or heat-induced allergen fragments was demonstrated for more than 50% of the identified allergens including tropomyosin. More interestingly, the identified prawn allergens were found to exist in oligomeric state using homology modelling. This property of allergenic proteins may enhance its IgE cross-linking capacity. Future cloning and sequencing of the novel prawn allergens and analysis of IgE binding to its recombinant forms will support future characterisation of the prawn allergens identified in this study.

A central objective of the experimental work conducted in this thesis was to demonstrate the effects of thermal processing of shellfish on the allergen stability, allergen-IgE interactions, allergen reactivity and allergen detection with particular emphasis on tropomyosin. In Chapter 2, Chapter 4 and Chapter 6, heat treatment of different shellfish species was demonstrated to enhance the IgE antibody reactivity of tropomyosin. In addition, an increase in IgE reactivity and cross-linking on the basophil cell surface was demonstrated, using functional assays to tropomyosin from heat-treated shellfish. These findings suggest heat-induced structural modifications to tropomyosin which may be responsible for enhanced access and affinity to IgE binding epitopes. Furthermore, Chapter 4 investigated the effects of thermal processing on patient IgE binding to novel prawn allergens. Two novel putative allergens, fructose bisphosphate aldolase and triose-phosphate isomerase demonstrated patient IgE binding, which was abolished after heat treatment; signifying the importance of characterising allergens in both raw and heat-treated shellfish.

An important aspect of the major shellfish allergen tropomyosin is the occurrence of immunological and clinical cross-reactivity due to its highly conserved primary structure. Patient IgE cross-reactivity between prawn and crab tropomyosin was demonstrated in Chapter 5 and 6. In addition, IgE cross-linking and degranulation was demonstrated for whole blood basophils in prawn allergic subjects, by both prawn and crab tropomyosin. More importantly, species-specific differential IgE binding was observed to tropomyosin. Thirty percent of patients elicited prawn-specific IgE binding to King prawn but not to Black tiger prawn tropomyosin, and 20% patients demonstrated IgE binding to crab but not to prawn tropomyosin. This may be attributed to the discussed differences in the primary structure of IgE binding regions of individual tropomyosins.

Using published amino acid sequences of allergenic tropomyosins, an extensive multiple sequence alignment analysis revealed IgE epitope-specific variations, not only among different invertebrate groups such as shellfish, insects and house dust mites, but also intra-group variations between crustaceans and molluscs. Interestingly, higher amino acid sequence identity was observed between crustaceans and dust mites as compared to

crustaceans and molluscs. Future work will need to correlate epitope-specific IgE cross-reactivity to the observed clinical cross-reactivity, especially in house dust mite allergic children.

In this thesis, a patient cohort was analysed for IgE binding reactivity to different allergens from various shellfish species. In Chapter 4, 5 and 6, tropomyosin-specific IgE binding was demonstrated in almost 50% of the patient cohort for three different crustacean species. Several studies have attempted to investigate the use of tropomyosin-specific IgE as a predictor of shrimp allergy. Gamez *et al* in 2011 deduced a positive predictive value of only 0.77 for tropomyosin. The experimental data outlined in this thesis supports previous findings that tropomyosin is the major IgE binding allergen. However, strong IgE binding was observed to other crustacean allergens. More interestingly, this study demonstrated patient mono-reactivity to crustacean allergens other than tropomyosin as demonstrated for the 70 kDa titin fragment in Black tiger prawns. This highlights the importance of analysing IgE binding to the whole allergen repertoire in contrast to a single major allergen; in this case, tropomyosin. Nevertheless, strong IgE binding reactivity to tropomyosin was observed in over 60% of the patient cohort.

A final aspect of this thesis, was the design and development of novel chemical-based and antibody-based analytical tools for the specific and sensitive detection and quantification of major shellfish allergens. In Chapter 2, a commercial monoclonal antibody was analysed for its tropomyosin-specific binding in 18 shellfish species. The mAb was able to detect all the analysed crustacean tropomyosins, however only 4 of the 7 analysed mollusc species. In Chapter 3, a novel mass spectrometric-based approach was established for the identification of major and minor shellfish allergens. Furthermore, a specific signature peptide was developed for prawn tropomyosin, which has applications for the quantification of trace allergens in packaged food. In Chapter 7, a highly sensitive antibody-based immunoassay was developed and validated, which involved recombinant prawn tropomyosin (Chapter 2) as a standard and a representative biomarker for whole crustacean allergen exposure. Using this immunoassay, aerosolised tropomyosin was quantified in the personal breathing zones of workers from crab-processing facilities. High

concentrations of air-borne tropomyosin was observed in activities involving the handling of cooked meat. The outcome of this particular study has implications in better management of occupational health and safety of seafood workers in this industry.

**Future direction:**

The experimental data outlined in this thesis extensively characterises major allergens from some of the most important shellfish species including its IgE binding properties, protein stability, IgE cross-reactivity and various allergen detection strategies. The findings of this thesis provide a platform for the development of improved diagnostic approaches for shellfish allergy. Future advances in this field of research may be pursued in various aspects as follows.

a) Allergen-specific IgE antibody detection in adults and children - Previous studies have demonstrated a differential IgE binding in children as compared to adults to different food allergens; for e.g. antibody reactivity in children to the allergen myosin light chain from Vannamei prawn. However, an extensive analysis investigating the presence of allergen-specific IgE in children has not been performed for all characterised shellfish allergens. In this thesis, the allergen-IgE binding analysis was performed using serum from adults only, with confirmed clinical reactivity to shellfish; and allergen-specific binding was demonstrated. Future studies involving the comparative IgE binding analysis, from adults and children, to the various shellfish allergens may provide an insight into the differential IgE binding in the two populations; which may lead to the development of a children-specific allergen panel for *in vitro* IgE quantification.

b) Component Resolved Diagnosis (CRD) for shellfish allergy – Previous studies involving the characterisation of allergens from various food sources have demonstrated improved accuracy in diagnosing allergy when quantifying specific-IgE against single allergen components, as compared to whole protein extracts. For example, improved accuracy in diagnosing peanut allergy was demonstrated, using *Ara h 2*-specific IgE quantification. Previous studies have investigated the use of tropomyosin-specific IgE as an accurate diagnostic tool. However, the work outlined in this thesis has demonstrated specific IgE

reactivity to additional characterised allergens. Future work involving the cloning and generation of a recombinant allergen panel for *in vitro* IgE quantification studied with a larger patient cohort will aid in the development of Component Resolved Diagnostics. The improved accuracy may result in decreased application of oral food challenges for crustacean allergy.

c) Heat-induced structural modifications of allergens and effects on gastric digestion – The work conducted in this thesis demonstrated heat-induced enhancement of IgE binding properties of shellfish allergens, particularly tropomyosin. Altered allergenicity as well as digestibility of food allergens has been attributed to formation of advanced glycation end (AGE) product formation through the Maillard reaction. Future work in the generation of AGE-products using purified recombinant shellfish allergens is essential in understanding its effects on IgE reactivity. Simulated gastric digestion of AGE-allergens and subsequent analysis of structural changes will provide a detailed insight into the altered digestibility and reactivity of the modified allergens.

d) Epitope mapping and development of hypoallergenic mutants for novel immunotherapeutic approaches – Structural elucidation of the IgE binding epitopes is essential in order to better understand the IgE antibody interaction with allergenic proteins and to deduce its correlation with clinical and epidemiological data. Future characterisation of the shellfish allergens identified in this thesis should involve the mapping of linear and conformational IgE epitopes. This can be performed using overlapping synthetic peptides, random-peptide phage display libraries or X-ray crystallographic approaches.

The development of effective vaccines for allergen-specific immunotherapy (SIT) requires the preservation of the immunogenicity of the allergen, but reduced IgE antibody binding capacity. Current allergen-specific immunotherapy involves repetitive injections of high doses of the allergen, which over a long period of time, restores normal tolerance towards the allergen. However, in the case of potent food allergens such as shellfish, fish, and peanuts, this entails a risk of inducing severe allergic reactions on exposure to such high doses.

The development of hypoallergenic shellfish allergens will assist in the development of safer immunotherapeutic approaches by reducing or abolishing the IgE reactivity, but preserving the T-cell reactivity. Different strategies such as heat-denaturation of proteins or destruction of the conformation by breaking disulphide bonds is not feasible in the case of shellfish allergens, since these proteins are heat-stable as demonstrated in the experimental work outlined in this thesis. Moreover, shellfish tropomyosin does not contain cystine residues and subsequently does not form disulphide bridges in its secondary or tertiary structure.

IgE epitope mapping and subsequent structural identification provides information to form a strategy to modify the allergenic protein to render it low- or non-IgE binding. This can be achieved by the identification of key amino acids in the IgE binding epitopes of the allergen and subsequent mutation to an uncharged amino acid using site-directed mutagenesis.

The experimental work and data analysis presented in this thesis generated substantial knowledge for the development of improved *in vitro* allergy diagnostic platforms and is the foundation for the future development of novel immunotherapeutic approaches for the treatment of crustacean allergy.

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## APPENDIX A

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### BUFFERS AND SOLUTIONS

#### A1.1 General Buffers

##### Phosphate buffered Saline (2L)

NaCl – 16 g

KCl – 0.4 g

Na<sub>2</sub>HPO<sub>4</sub> – 2.88 g

KH<sub>2</sub>PO<sub>4</sub> – 0.48 g

ddH<sub>2</sub>O – upto 1L

pH adjusted to pH 7.4 using 6M HCL and autoclaved

##### PBS-T wash buffer

1X PBS with 0.05% Tween-20

##### TAE Buffer (50X Stock)

Tris-HCl – 242 g/L

Glacial acetic acid – 57.1 mL

0.5 M EDTA, pH 8.0 – 100 mL

ddH<sub>2</sub>O upto 1000 mL and autoclaved

#### A1.2 SDS-PAGE Solutions

##### Solution B

2M Tris-HCl, pH 8.8 – 75 mL

10% SDS in ddH<sub>2</sub>O – 4 mL

ddH<sub>2</sub>O – 21 mL

##### Solution C

1M Tris-HCl, pH 6.8 – 50 mL

10% SDS in ddH<sub>2</sub>O – 4 mL

ddH<sub>2</sub>O – 46 mL

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5X Protein sample loading buffer

0.6 mL 1M Tris-HCl, pH 6.8 – 60 mM

5mL 50% Glycerol – 25%

2 mL 10% SDS – 2%

Dithiothreitol – 100 mM

1 mL 1% Bromophenol blue – 0.1%

ddH<sub>2</sub>O – upto 10 mL

12% SDS-PAGE gel recipe

Resolving gel (for 20mL, 4 minigels)

40% 29:1 Acrylamide – 6 mL

Solution B – 5 mL

ddH<sub>2</sub>O – 8.9 mL

10% Ammonium persulphate – 100 µL

TEMED – 10 µL

Stacking gel (for 10mL, 4 minigels)

40% 29:1 Acrylamide – 0.93mL

Solution C – 2.5 mL

ddH<sub>2</sub>O – 6.5 mL

10% Ammonium persulphate – 100 µL

TEMED – 10 µL

1X Gel Electrophoresis running buffer

Tris – 3 g/L

Glycine – 14.4 g/L

SDS – 1 g/L

ddH<sub>2</sub>O – upto 1000 mL

SDS-PAGE gel destaining solution

Methanol (AR grade) – 500mL

Glacial acetic acid – 100mL

ddH<sub>2</sub>O – 400 mL

### **A1.3 Immunoblotting Buffers**

#### Transfer buffer

Tris – 1.164 g

Glycine – 0.58 g

10% SDS – 750  $\mu$ L

Methanol – 40 mL

ddH<sub>2</sub>O – upto 200mL

#### Blocking buffer

Skimmed Milk powder – 5g

PBS-T – upto 100mL

#### Antibody dilution buffer

Skimmed Milk powder – 1g

PBS-T – upto 100mL

### **A1.4 Protein Purification Buffers**

30 mM Acetate buffer, pH 5.5 (Binding buffer)

Glacial acetic acid – 0.27 g/L

Sodium acetate (trihydrate) – 3.46 g/L

ddH<sub>2</sub>O – upto 1000 mL

30 mM Acetate buffer, 1M NaCl pH 5.5 (Elution buffer)

Glacial acetic acid – 0.27 g/L

Sodium acetate (trihydrate) – 3.46 g/L

Sodium chloride – 58 g/L

ddH<sub>2</sub>O – upto 1000 mL

### **A1.5 Molecular Biology Solutions**

#### LB broth

Tryptone – 10 g/L

NaCl – 10 g/L  
Yeast extract – 5 g/L  
ddH<sub>2</sub>O – upto 1000 mL and autoclaved

LB plates

Tryptone – 10 g/L  
NaCl – 10 g/L  
Yeast extract – 5 g/L  
Agar – 15 g/L  
ddH<sub>2</sub>O – upto 1000 mL and autoclaved

Ampicillin stock

Ampicillin – 1 g  
70% Ethanol – 10 mL and filter sterilised

Glycerol stocks

Fresh overnight culture – 700 µL  
50% sterile glycerol in ddH<sub>2</sub>O – 300 µL

**A1.6 ELISA Buffers**

Coating buffer, pH 9.6

50 mM Sodium bicarbonate – 4.25 mL  
50 mM Sodium carbonate – 2 mL  
ddH<sub>2</sub>O – upto 25 mL

Well washing buffer

PBS with 0.05% Tween20

## APPENDIX B

### SUPPLEMENTARY TABLES AND FIGURES

#### Tables

**Table B1.1:** Start and end position numbers, calculated molecular weights and amino acid sequences of peptides identified for each IgE reactive tropomyosin using mass spectrometry

MW of protein from SDS-PAGE	Start-end	MW, calc.	Peptide sequence
38 kDa Tropomyosin molecule	67-74	914.07	ANIQLVEK
	153-160	950.02	FLAEEADR
	141-149	1061.22	MDALENQLK
	190-198	1129.28	IVELEEEELR
	67-76	1157.33	ANIQLVEKDK
	169-178	1146.32	LAMVEADLER
	92-101	1257.41	IQLLEEDLER
	168-178	1274.50	KLAMVEADLER
	113-125	1376.40	LAEASQAADESER
	91-101	1413.59	RIQLLEEDLER
	77-90	1414.54	ALSNAEGEVAALNR
	252-260	1587.70	EVDRLEDELVNEK
	169-182	1631.82	LAMVEADLERAEER
	92-105	1758.90	IQLLEEDLERSEER
	16-30	1748.84	DNAMDRADTLEQQNK
26 kDa Tropomyosin fragment	67-74	914.07	ANIQLVEK
	153-160	950.02	FLAEEADR
	141-149	1061.22	MDALENQLK
	190-198	1129.28	IVELEEEELR
	169-178	1146.32	LAMVEADLER
	67-76	1157.33	ANIQLVEKDK
	169-178	1146.32	LAMVEADLER
	168-178	1274.50	KLAMVEADLER
	256-264	1345.47	LEDELVNEKEK
	113-125	1376.40	LAEASQAADESER
	77-90	1414.54	ALSNAEGEVAALNR
	252-260	1587.70	EVDRLEDELVNEK
	169-182	1631.82	LAMVEADLERAEER
	75-90	1657.8	DKALSNAEGEVAALNR
	113-126	1663.78	LAEASQAADESERMR
16-30	1748.84	DNAMDRADTLEQQNK	
92-105	1758.90	IQLLEEDLERSEER	
134-149	1878.04	SLSDEERMDALENQLK	

**Table B1.2:** A list of generated peptides of TM extracted from BTP that have been generated by this study using different enzymes and MS ion sources.

Protease	Residues position	Peptide sequence (Z)	Molecular weight		Ion Source
			Calculated	Expected	
Trypsin	8-15	MQAMKLEK (2)	977.5038	977.4839	ESI
	16 – 30	DNAMDRADTLEQQNK (3)	1747.7737	1747.7795	
	22-35	ADTLEQQNKEANNR (2)	1747.7795	1747.7958	
	36 – 48	AEKSEEEVHNLQK (3)	1539.7529	1539.7462	
	39 – 48	SEEEVHNLQK (2)	1211.5782	1211.5673	
	39 – 49	SEEEVHNLQKR (2)	1367.6793	1367.6832	
	50 - 66	MQQLENDLDQVQESLLK (2)	2029.9990	2030.0026	
	67 - 76	ANIQLVEKDK (2)	1156.6452	1156.6454	
	77 - 90	ALSNAEGEVAALNR (2)	1413.7212	1413.7342	
	77 - 91	ALSNAEGEVAALNRR (2)	1569.8223	1569.8431	
	91 - 101	RIQLLEEDLER (2)	1412.7623	1412.7934	
	102 -112	SEERLNTATTK (2)	1248.6310	1248.6226	
	106 -125	LNTATTKLAEASQAADER (3)	2105.0236	2105.0447	
	113 - 125	LAEASQAADER (2)	1375.6215	1375.6232	
	134 - 149	SLSDEERMDALENQLK	1876.8836	1876.8873	
	141 - 149	MDALENQLK (2)	1060.5223	1060.5236	
	153 - 160	FLAEEADR (2)	949.4505	949.4376	
	168 - 178	KLAMVEADLER (2)	1273.6700	1273.6709	
	169 - 182	LAMVEADLERAEER (2)	1630.7984	1630.8204	
	190 - 198	IVELEELR (2)	1128.6026	1128.6139	
	206 - 217	SLEVSEKANQR	1388.6895	1388.7188	
	218 - 226	EEAYKEQIK (2)	1136.5713	1136.5687	
	252 - 264	EVDRLDELVNEK (2)	1586.7787	1586.7966	
	267- 284	YKSITDELDTFSELSGY (2)	2094.9633	2094.9916	
	269 - 284	SITDELDTFSELSGY	1803.8051	1803.8475	
	Glu-C (V8)	3 - 14	AIKKKMQAMKLE (2)	1417.8148	
43 - 54		VHNLQKRMQQLE (2)	1522.8038	1522.8268	
63 - 73		SLLKANIQLVE (2)	1226.7234	1226.7528	
85 - 96		VAALNRRIQLE (2)	1394.8357	1394.8680	
85 - 97		VAALNRRIQLEE (2)	1523.8783	1523.8982	
105 - 115		RLNTATTKLAE (2)	1216.6775	1216.6927	
125 - 131		RMRKVLE (2)	930.5433	930.5576	
151 - 157		ARFLAEE (2)	834.4235	834.4310	
165 - 173		VARKLAMVE (2)	1015.5848	1015.5959	
197 - 208		LRVVGNNLKSLE (2)	1340.7776	1340.8081	
224 - 236		QIKTLTNKLKAAE (2)	1456.8613	1456.8909	
244 - 252		RSVQKLQKE (2)	1114.6458	1114.6608	
266 - 273		KYSITDE (2)	982.4971	982.5179	
Trypsin	16 - 30	DNAMDRADTLEQQNK	1747.7795	1747.7927	MALDI
	22 - 30	ADTLEQQNK	1747.7795	1747.7788	
	22 - 35	ADTLEQQNKEANNR	1629.7706	1629.7785	
	36 - 48	AEKSEEEVHNLQK	1539.7529	1539.6927	
	39 - 48	SEEEVHNLQK	1211.5782	1211.4927	

39 - 49	SEEEVHNLQKR	1367.6793	1367.5927	
50 - 66	MQQLENDLDQVQESLLK	2029.9990	2029.9927	
67 - 74	ANIQLVEK	913.5232	913.4396	
67 - 76	ANIQLVEKDK	1156.6452	1156.5927	
77 - 90	ALSNAEGEVAALNR	14137212	1413.6927	
91 - 101	RIQLEEDLER	1412.7623	1412.6927	
92 - 105	IQLEEDLERSEER	1757.8795	1757.7927	
102 - 112	SEERLNTATTK	1248.6310	1248.5582	
113 - 125	LAEASQAADER	1375.6215	1375.5927	
134 - 149	SLSDEERMDALENQLK	1876.8836	1876.7927	
141 - 149	MDALENQLK	1060.5223	1060.4927	
153 - 160	FLAEEADR	949.4505	949.3927	
153 - 161	FLAEEADRK	1077.5454	1077.5252	
162 - 168	YDEVARK	879.4450	879.4282	
168 - 178	KLAMVEADLER	1273.6700	1273.5927	
169 - 178	LAMVEADLER	1145.5750	1145.5927	
169 - 182	LAMVEADLERAEER	1630.7984	1630.7927	
190 - 198	IVELEEELR	1128.6026	1128.4927	
206 - 217	SLEVSEKANQR	1388.6895	1388.5927	
214 - 222	ANQREEAYK	1107.5308	1107.4927	
218 - 226	EEAYKEQIK	1136.5713	1136.4927	
232 - 238	LKAAEAR	757.4446	757.4322	
239 - 244	AEFAER	721.3395	721.2927	
252 - 264	EVDREDELVNEK	1586.7787	1586.6927	
269 - 284	SITDELDTFSELSGY	1803.8051	1803.7927	
2 - 15	DAIKKKMQAMKLEK (3)	1660.9368	1660.9815	
58 - 74	DQVQESLLKANIQLVEK (3)	1954.0734	1954.1163	
103-114	EERLNTATTKLA(2)	1345.7201	1345.7476	
121-130	DESERMKVL (2)	1261.6448	1261.6673	
150-158	EARFLAEEA (2)	1034.5032	1034.5286	
163-174	DEVARKLAMVEA (2)	1330.6915	1330.7083	ESI
184-194	ETGESKIVELE (2)	1232.6136	1232.6384	
195-207	EELRVVGNLKSL (2)	1469.8202	1469.8368	
223-235	EQIKTLTNKLKAA (2)	1456.8613	1456.8808	
243-253	ERSVQKLQKEV (2)	1342.7568	1342.7718	
275 - 284	DQTFSELSGY(2)	1145.4877	1145.5052	
2 - 15	DAIKKKMQA MKLEK	1660.9368	1660.8927	
58 - 74	DQVQESLLKANIQLVEK	1954.0735	1953.9927	
62 - 74	ESLLKANIQLVEK	1483.8609	1483.7927	
75 - 95	DKALSNAEGEVAALNRRIQ L	2280.2549	2280.1927	
82 - 97	EGEVAALNRRIQLEE	1838.9850	1838.8927	
103 - 114	EERLNTATTKLA	1345.7201	1345.6927	
142 - 149	DALENQLK	929.4818	929.3927	
150 - 158	EARFLAEEA	1034.5032	1034.3927	
163 - 174	DEVARKLAMVEA	1330.6915	1330.5927	
195 - 207	EELRVVGNLKS	1346.6864	1346.5927	
223 - 235	EQIKTLTNKLKAA	1456.8613	1456.7927	
236 - 242	EARAFA	792.3766	792.2927	
243 - 253	ERSVQKLQKEV	1342.7568	1342.6927	
258 - 271	DELVNEKEYKSIT	1694.7826	1694.7927	
275 - 284	DQTFSELSGY	1145.4877	145.3927	MALDI

**Table B 1.3:** Amino acid sequences of peptides identified for each IgE reactive protein using mass spectrometry, with accession numbers for identified proteins using GenBank database.

Band no.	Mass (kDa)	Peptide sequence	Database no.	Protein identity	Score	Identified allergen
<b>Prawn-Raw (P-raw)</b>						
1	70	AGQIINLVTR IELSVQMKK	AB055927.1	Titin	57	-
2	42	AVFDQLKEK VSSTLSSLEGELK LIDDFLFLK IISMQMGGDLGQVFR LTSAVNEIEK GTRGEHTEAEGGIYDISNK MGLTEFQAVK EMQDGILELIK	GQ246164	Arginine kinase	461	Pen m 2
		ALQASVLR IANAIAPGK GILAADESVMGK LKDIGVENTEENR GILAADESVMGKR RIANAIAPGK KPWALTFSYGR ETPSYLGMLENANVLAR	Q9URB4	Aldolase	268	Cand a FBA
3	38.0	FLQAANACR AVFDQLKEK LTSAVNEIEK MGLTEFQAVK LIDDFLFLK GTYYPVTGMSK VSSTLSSLEGELK IISMQMGGDLGQVFR GEHTEAEGGIYDISNK	GQ246164	Arginine kinase	485	Pen m 2
		IVELEELR ANIQLVEKDK IQLLEEDLER ALSNAEGEVAALNR	HM486525	Tropomyosin	253	Pen m 1
4	35	AVFDQLK FLQAANACR AVFDQLKEK LTSAVNEIEK MGLTEFQAVK LIDDFLFLK GTYYPVTGMSK LTSAVNEIEKR EMQDGILELIK VSSTLSSLEGELK IISMQMGGDLGQVFR GEHTEAEGGIYDISNK LGFLTFCPTNLGTTVR GEHTEAEGGIYDISNKR GTRGEHTEAEGGIYDISNK	GQ246164	Arginine kinase	961	Pen m2
4	26.5	AGGLTLER VGLDEYR VFIANQFK NDFECLAVR DGEVTVDFEK GEFSADAYANNQK YMYDIDNNGFLDK AIDVNGDGKVGLEDEYR YMYDIDNNGFLDKNDFECLAVR	FJ184279	Sarcoplasmic calcium protein	437	Lit v 4
5	16.8	KFFVGGNWK GAFTGEISPAMVK TQEVVFAQMK AAIDGIIISFMK	FJ462738	Triose-phosphate isomerase	128	Cra c 8
<b>Prawn-Whole Heated (P-WH)</b>						
1	70	AGQIINLVTR IELSVQMKK	AB055927.1	Titin fragment	56	-
2	38	ANIQLVEK FLAEEADR MDALENQLK IVELEELR ANIQLVEKDK LAMVEADLER IQLLEEDLER KLAMVEADLER LAEASQAADER RIQLLEEDLER ALSNAEGEVAALNR EVDRLLEDLVNEK LAMVEADLERAEER IQLLEEDLERSEER DNAMDRADTLEQQNK SLSDEERMDALENQLK	HM486525	Tropomyosin	910	Pen m1

3	36.5	LIDHFLFK TVTAVNEIEK	GQ246164	Arginine kinase	77	Pen m 2
	33.5	ANIQLVEK FLAEEADR MDALENQLK IVELEEELR LAMVEADLER ANIQLVEKDK LAMVEADLER IQLLEEDLER KLAMVEADLER LEDELVNEKEK LAEASQAASESER ALSNAEGEVAALNR EVDRLLEDELVNEK LAMVEADLERAEER DKALSNAEGEVAALNR LAEASQAASESERMR DNAMDRADTLEQQNK IQLLEEDLERSEER DNAMDRADTLEQQNK SLSDEERMDALENQLK SLQDEERMDALENQLK	HM486525	Tropomyosin	961	Pen m 1
4	26.5	ANIQLVEK FLAEEADR MDALENQLK IVELEEELR LAMVEADLER ANIQLVEKDK LAMVEADLER KLAMVEADLER LEDELVNEKEK LAEASQAASESER ALSNAEGEVAALNR EVDRLLEDELVNEK LAMVEADLERAEER DKALSNAEGEVAALNR LAEASQAASESERMR DNAMDRADTLEQQNK IQLLEEDLERSEER SLQDEERMDALENQLK SLSDEERMDALENQLK	HM486525	Tropomyosin	1187	Pen m 1
5	16.8	GTFDEIGR EGFQLMDR	ADM34185	Myosin light chain	79	Lit v 3
		VGLDEYR VFIANQFK DGEVTVDFEK GEFSADAYANNQK	FJ184279	Sarcoplasmic calcium protein	165	Lit v 4

**Table B1.4:** Demographics of the 10 shellfish allergic patients and one non-atopic control donor. Total IgE and shrimp-specific IgE (f24) were quantified using ImmunoCAP

Subject	Gender	Age	Total IgE (kU/L)	Shrimp IgE (f24) (kU/L)	Shellfish exposure	Asthma	Rhinitis
1	F	24	372	1.82	Oral	-	-
2	F	17	242	1.32	Contact	+	+
3	M	34	55	0.37	Oral	+	-
4	F	65	150	0.16	Oral	+	+
5	F	56	440	0.50	Oral	-	-
6	F	33	28	0.21	Contact	-	+
7	M	23	158	0.85	Oral	-	+
8	M	46	822	19.7	Oral	+	+
9	M	21	283	5.37	Oral	-	-
10	F	23	1946	9.5	Oral	-	+

**Table B1.5:** Scientific names and Genbank accession numbers of the full length tropomyosin sequences used to create the phylogenetic tree and the epitope specific multiple sequence alignment.

Scientific name	Common Name	Genbank accession number
<i>Crangon crangon</i>	Brown shrimp	GI:238477263
<i>Pandalus borealis</i>	Northern shrimp	GI:312831088
<i>Pandalus eous</i>	Alaskan pink shrimp	GI:125995161
<i>Macrobrachium rosenbergii</i>	Giant freshwater prawn	GI:288819271
<i>Farfantepenaeus aztecus</i>	Northern brown shrimp	GI:73532979
<i>Fenneropenaeus chinensis</i>	Chinese white shrimp	GI:281484502
<i>Litopenaeus vannamei</i>	Vannamei prawn	GI:170791252
<i>Marsupenaeus japonicus</i>	Kuruma prawn	GI:125995159
<i>Penaeus monodon</i>	Black tiger prawn	GI:304441897
<i>Squilla oratoria</i>	Japanese mantis shrimp	GI:148285611
<i>Squilla aculeate</i>	Mantis shrimp	GI:40548517
<i>Euphausia pacifica</i>	North pacific krill	GI:156712754
<i>Euphausia superba</i>	Antarctic krill	GI:156712752
<i>Homarus americanus</i>	American lobster	GI:14285796
<i>Paralithodes camtschaticus</i>	Red king crab	GI:125995165
<i>Portunus sanguinolentus</i>	Spotted crab	GI:119674937
<i>Portunus trituberculatus</i>	Swimming crab	GI:151505281
<i>Scylla serrata</i>	Mud crab	GI:151505279
<i>Eriocheir sinensis</i>	Chinese mitten crab	GI:134305330
<i>Erimacrus isenbeckii</i>	Hair crab	GI:125995171
<i>Erimacrus isenbeckii</i>	Hair crab	GI:125995169
<i>Chionoecetes opilio</i>	Snow crab	GI:125995167
<i>Locusta migratoria</i>	Locusts	GI:227691
<i>Blattella germanica</i>	German cockroach	GI:8101069
<i>Periplaneta Americana</i>	American cockroach	GI:239740599
<i>Periplaneta fuliginosa</i>	Smoky brown cockroach	GI:19310971
<i>Dermatophagoides farinae</i>	American house dust mite	GI:42559584
<i>Dermatophagoides pteronyssinus</i>	European house dust mite	GI:208970286
<i>Anisakis simplex</i>	Herring worm	GI:350285785
<i>Caenorhabditis elegans</i>	Roundworm	GI:9966517
<i>Ommastrephes bartramii</i>	Neon flying squid	GI:83715934
<i>Sepioteuthis lessoniana</i>	Bigfin reef squid	GI:83715930
<i>Sepia esculenta</i>	Golden cuttlefish	GI:83715928
<i>Sepia officinalis</i>	Common cuttlefish	GI:332672682
<i>Todarodes pacificus</i>	Pacific squid	GI:83715932
<i>Loligo bleekeri</i>	Bleekers squid	GI:290794772
<i>Octopus vulgaris</i>	Octopus	GI:83715936
<i>Argopecten irradians</i>	Atlantic bay scallop	GI:60892794

<i>Mimachlamys nobilis</i>	Noble scallop	GI:9954253
<i>Chlamys nipponensis</i>	Japanese scallop	GI:4062999
<i>Mizuhopecten yessoensis</i>	Giant scallop	GI:11177141
<i>Haliotis diversicolor</i>	Abalone	GI:9954249
<i>Haliotis rufescens</i>	Red abalone	GI:407417
<i>Haliotis discus discus</i>	Disk abalone	GI:219806586
<i>Haliotis asinina</i>	Donkey ear abalone	GI:32492443
<i>Biomphalaria glabrata</i>	Freshwater snail	GI:155942
<i>Helix aspersa</i>	Garden snail	GI:4468224
<i>Neptunea polycostata</i>	Whelk	GI:219806590
<i>Mytilus galloprovincialis</i>	Mediterranean mussel	GI:1843405
<i>Mytilus edulis</i>	Blue mussel	GI:1086579
<i>Perna viridis</i>	Green mussel	GI:9954251
<i>Crassostrea gigas</i>	Pacific oyster	GI:219806594
<i>Scapharca broughtonii</i>	Blood clam	GI:219806592
<i>Tegillarca granosa</i>	Blood cockle	GI:359326555
<i>Fulvia mutica</i>	White cockle	GI:219806596
<i>Ruditapes philippinarum</i>	Manila clam	GI:219806573
<i>Solen strictus</i>	Goulds razor shell	GI:219806602
<i>Meretrix lyrata</i>	white clam	GI:359326551
<i>Meretrix meretrix</i>	Asiatic hard clam	GI:359326553
<i>Pseudocardium sachalinensis</i>	Surf clam	GI:219806598
<i>Tresus keenae</i>	Horse clam	GI:219806600

## Figures

		Crustaceans											Molluscs							
		Specimen no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Crustaceans	Black tiger prawn	1	100																	
	King prawn	2	95	100																
	Vannamei prawn	3	100	95	100															
	Banana prawn	4	100	95	100	100														
	Green tiger prawn	5	•	•	•	•	•													
	Blue swimmer crab	6	97	94	97	97	•	100												
	Sand crab	7	•	•	•	•	•	•	•											
	Snow crab	8	89	88	89	89	•	88	•	100										
	Slipper lobster	9	97	95	97	97	•	99	•	88	100									
	Rock lobster	10	95	100	95	95	•	94	•	88	95	100								
	Yabby	11	98	95	98	98	•	98	•	89	98	95	100							
Molluscs	Green mussel	12	55	57	55	55	•	55	•	54	55	57	55	100						
	Blue mussel	13	57	58	57	57	•	56	•	56	56	58	57	94	100					
	Scallop	14	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	Oyster	15	61	62	61	61	•	61	•	59	61	62	61	78	78	•	100			
	Sea snail	16	60	61	60	60	•	60	•	58	60	61	60	68	72	•	73	100		
	Octopus	17	63	64	63	63	•	63	•	63	63	64	63	69	70	•	76	76	100	
	Squid	18	62	63	62	62	•	62	•	62	63	63	63	70	71	•	75	76	91	100

**Figure B 2.1:** Amino acid sequence homology data for tropomyosin from crustacean and mollusc species. The protein sequences have been extracted from Genbank (<http://www.ncbi.nlm.nih.gov/genbank>); accession numbers have been listed in table 2.1.

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-----10 -----20 -----30 -----40 -----50 -----60
MDAIKKKMQA MKLEKDNAMD RADTLEQQNK EANNRAEKSE EEVHNLQKRM QQLENDLLDQV
-----70 -----80 -----90 -----100 -----110 -----120
QESLLKANIQ LVEKDKALSN AEGEVAALNR RIQLLEEDLE RSEERLNTAT TKLAEASQAA
-----130 -----140 -----150 -----160 -----170 -----180
DESERM RKVL ENRSLSDEER MDALENQLK EAREFLAEEADR KYDEVARKLA MVEADLERAE
-----190 -----200 -----210 -----220 -----230 -----240
ERAETGESKI VELEEEELRVV GNNLKSLEVS EEKANQREEA YKEQIKTLTN KLKAAEARAE
-----250 -----260 -----270 -----280 -----284
FAERSVQKLQ KEVDRLEDEL VNEKEKYKSI TDFLDQTFSE LSGY

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No.	Amino acid sequence of peptide fragments derived from the 26 kDa protein
1	ANIQLVEK
2	FLAEEADR
3	IVELEELR
4	MDALENQLK
5	ANIQLVEKDK
6	LAMVEADLER
7	LEDELVNEKEK
8	KLAMVEADLER
9	LAEASQAADSESR
10	EVDRLLEDELVNEK
11	IQLLEEDLERSEER
12	ALSNAEAGEVAALNR
13	LAMVEADLERAEER
14	LAEASQAADSESRMR
15	DNAMDRADTLEQQNK
16	SLQDEERMDALENQLK
17	SLSDEERMDALENQLK
18	DKALSNAEAGEVAALNR

**Figure B 2.2:** Amino acid sequence of Black tiger prawn tropomyosin. The peptide fragments derived from the 26 kDa protein by tryptic digest were identified by mass spectrometric analysis and are highlighted in different colors. The proposed eight IgE antibody binding epitopes by Reese et al. 2005 are boxed and numbered from I to VIII.

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## APPENDIX C

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### PUBLICATIONS

- C1.1** Lopata AL, Kamath S. Shellfish Allergy Diagnosis - Gaps and Needs. *Current Allergy & Clinical Immunology* 2012; 25:60-6.
- C1.2** Kamath SD, Abdel Rahman AM, Komoda T, Lopata AL. Impact of heat processing on the detection of the major shellfish allergen tropomyosin in crustaceans and molluscs using specific monoclonal antibodies. *Food Chemistry* 2013; 141:4031-9.
- C1.3** Abdel Rahman AM, Kamath S, Lopata AL, Helleur RJ. Analysis of the allergenic proteins in black tiger prawn (*Penaeus monodon*) and characterization of the major allergen tropomyosin using mass spectrometry. *Rapid Communications in Mass Spectrometry* 2010; 24:2462-70.
- C1.4** Kamath SD, Rahman AM, Voskamp A, Komoda T, Rolland JM, O'Hehir RE, Lopata AL. Effect of heat processing on antibody reactivity to allergen variants and fragments of black tiger prawn: A comprehensive allergenomic approach. *Mol Nutr Food Res* 2014; 58:1144-55.
- C1.5** Abramovitch JB\*, Kamath S\*, Varese N, Zubrinich C, Lopata AL, O'Hehir RE, Rolland JM. IgE Reactivity of Blue Swimmer Crab *Portunus pelagicus* Tropomyosin, *Por p 1*, and Other Allergens; Cross-Reactivity with Black Tiger Prawn and Effects of Heating. *PLoS ONE* 2013; 8:e67487. \*(Equal first authors)
- C1.6** Kamath SD, Thomassen MR, Saptarshi SR, Nguyen HM, Aasmoe L, Bang BE, Lopata AL. Molecular and immunological approaches in quantifying the air-borne food allergen tropomyosin in crab processing facilities. *International Journal of Hygiene and Environmental Health* 2014; 217:740-50.